

INDIAN AGRICULTURAL  
RESEARCH INSTITUTE, NEW DELHI.







# **BIOCHEMICAL JOURNAL**

EDITED FOR THE BIOCHEMICAL SOCIETY

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**VOLUME XXII 1928**

**PART II, pp. 777 to end**

**CAMBRIDGE**

**AT THE UNIVERSITY PRESS**

**1928**



# XCVIII. THE ULTRA-VIOLET ABSORPTION SPECTRUM OF CHLOROPHYLL IN ALCOHOLIC SOLUTION.

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*(Received May 3rd, 1928.)*

ATTENTION was drawn to the ultra-violet absorption spectrum of chlorophyll as a corollary to the spectrographic examination of olive oil [Lewkowitsch, 1927]. The latter gave distinct indications of selective absorption with a maximum at a wave-length 2670 Å.v., and the question arose whether this band was caused by chlorophyll, which in the visible range displays its own characteristic bands strongly marked in the absorption spectrum of olive (and some other vegetable) oils [Marcille, 1910].

The ultra-violet absorption spectrum of chlorophyll is unrecorded in the list of absorption spectra of organic compounds issued by the British Association in 1916, and the only account in the literature is a paper by Dhéré and Rogowski [1912]. These workers examined  $\alpha$ - and  $\beta$ -“natural chlorophyll” from *Tarax baccata* and crystalline chlorophyll (from *Galeopsis tetrahit* by the combined methods of Monteverde and Willstätter) in solution in anhydrous ether; they obtained evidence of absorption maxima at  $\lambda$  4300 and  $\lambda$  3000–3100 Å.v. The method employed was to photograph the spectra transmitted through increasing thicknesses of solution.

Vlès [1919] calculated the anticipated spectrum in the ultra-violet of chlorophyll from an expression of the type  $X = X_0 + An + Bn^2 + Cn^3$  from which he deduced the absorption band at 4320 Å.v.; his formula, however, did not provide for the band found by Dhéré at 3000 Å.v.

For the purposes of the present investigation a solution of chlorophyll extracted in the cold from dry powdered nettle leaves by alcohol was used. The solvent was purified for spectrographic purposes from absolute alcohol by distillation over iodine followed by distillation from excess of zinc dust. The solvent so prepared had practically no absorption down to a wave-length of about 2500 Å.v.; below this point the general absorption of the alcohol hindered examination of the chlorophyll.

The concentrated chlorophyll solution, stored in the dark, was used as a stock from which the more dilute solutions actually examined in the spectrophotometer were prepared immediately before use. Such a diluted solution was examined within one day, and the portion exposed to the light in the test-tube of the photometer was frequently renewed; these precautions were found necessary as the dilute solutions became rapidly decolorised, especially under the influence of the light of the spark.

The spectra were recorded by means of a Bellingham and Stanley spectrophotometer [Prideaux, 1926; Stanley, 1927] in conjunction with a quartz spectrograph. A condensed spark between iron electrodes was used as a source of ultra-violet light.

The spectrum obtained is shown in Fig. 1, in which "density,"  $\log$ . (incident light/transmitted light), is plotted against wave-length in Å.U. As the solutions examined were necessarily of arbitrary concentration, the figures for density are only relative values.

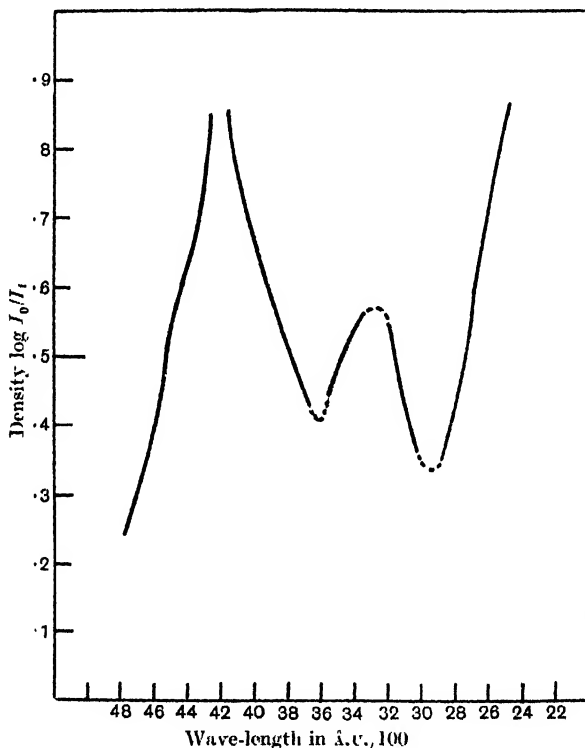


Fig. 1. Absorption spectrum of nettle chlorophyll in alcoholic solution.

Absorption maxima are shown at 4200 and 3250 Å.U., figures which agree reasonably with those of Dhéré; the slight difference in position of the maxima might be due to the effect of the different solvent used [Vogel, 1877; Van Gulik, 1915]. No trace could be found of any further minimum or descent of the curve which rose steeply below a wave-length of 2400 Å.U.

#### REFERENCES.

- Dhéré and Rogowski (1912). *Compt. Rend. Acad. Sci.* **155**, 653.  
 Lewkowitsch (1927). *J. Soc. Chem. Ind.* 195 T.  
 Marcille (1910). *Ann. Falsif.* **131**, 43.  
 Prideaux (1926). *J. Soc. Chem. Ind.* 644.  
 Stanley (1927). *J. Actinotherapy*, Dec., 174.  
 Van Gulik (1915). *Ann. Physik.* **46**, 152.  
 Vlès (1919). *Compt. Rend. Acad. Sci.* **168**, 1044.  
 Vogel (1877). *Prakt. Spektralanalyse*, 299.

## XCIX. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VII.

By SYLVESTER SOLOMON ZILVA.

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*Report to the Medical Research Council.*

*(Received May 1st, 1928.)*

In a previous contribution of this series [Zilva, 1927, 2] it was shown that the antiscorbutic activity in decitrated lemon juice is associated with a capacity for reducing phenolindophenol to its leuco-base. It was also found that when the juice had been heated in an autoclave at 120° under strictly anaerobic conditions for 1 hour, the antiscorbutic potency and the reducing capacity of the juice disappeared much more quickly on storage than it usually did in a similar juice which had not been previously autoclaved and which was stored under the same conditions. This phenomenon has since been subjected to further investigation in order to throw more light on the subject and some of the results are recorded in this communication.

### EXPERIMENTAL.

The method of testing was the same as previously described. Three guinea-pigs weighing 250-300 g. were used for each dose, and doses equivalent to 1.5, 3 and 5 cc. of the original lemon juice were usually employed. The administration of the daily dose began after the animals had subsisted on the basal diet for 14 days. In the case of positive tests the guinea-pigs were chloroformed 60 days after the commencement of the experiment and the degree of scurvy, if present, assessed at the autopsy. With negative preparations, the animals, as is invariably the case on a basal diet of oats, bran and autoclaved milk, succumbed to scurvy within a month.

#### *The instability of the antiscorbutic factor in the absence of the reducing agency.*

When phenolindophenol is added to decitrated lemon juice until it is no longer reduced, the solution becomes alkaline owing to the hydrolysis of the indicator. The quick deterioration of the antiscorbutic activity in such solution on storage could, therefore, be ascribed either to accelerated oxidation in alkaline medium [Zilva, 1923] or to the removal of the protective action of the reducing principle. In order to test the second hypothesis it

was consequently necessary to adjust the reaction to neutrality very soon after the addition of the phenolindophenol. This is technically complicated by the fact that the electrometric measurement of the  $p_H$  in the adjustment of the reaction is tedious and long, especially as the presence of  $CO_2$  and of carbonate in the medium requires the passing of hydrogen through the electrode for a considerable time before equilibrium is established. On the other hand, the use of  $p_H$  indicators in the adjustment of the reaction is complicated by the presence of phenolindophenol in the solution. This difficulty was, however, overcome in the following way. Ordinary decitrated lemon juice was adjusted to  $p_H$  7. Solid phenolindophenol was then added cautiously until the indicator was no longer decolorised, *avoiding the addition of excess*. The  $p_H$  of the solution at this stage was approximately 8.4. A citric acid solution was now introduced without delay, drop by drop, until the  $p_H$  fell to 7, utilising simultaneously the comparator and capillator methods with phenol red as indicator. With practice, a fair degree of accuracy could thus be obtained. One portion of decitrated lemon juice treated in this way was fed *at once* to the guinea-pigs whilst another portion was stored in the *neutral condition* in the cold room for 24 hours before it was administered to the animals. All the 9 animals which received the stored preparation died of scurvy within a month. The control animals were chloroformed after 43 days, some signs of scurvy being established in the guinea-pigs on the lower doses. Excluding the detrimental action of the indicator on the vitamin *per se* this comparatively rapid inactivation of the decitrated lemon juice on storage can only be ascribed to the disappearance of the reducing activity, since untreated decitrated lemon juice stored under the same conditions at about  $p_H$  7 for 24 hours loses comparatively little of its activity. The control experiment showed that the juice was active before storage. The animals in this set were chloroformed after 13 days as it has already been shown [Zilva, 1927, 2] that this treatment with phenolindophenol does not destroy the potency to any significant extent if tested immediately. The condition of the animals at the time of chloroforming showed that this was also the case in this instance. The post-mortem examination lent further confirmation of the activity of the preparation.

*The stability of the antiscorbutic potency of comparatively pure fractions.*

If the assumption is justified that in the chemical fractionation of the antiscorbutic factor substances are removed which contribute to the stability of the active principle, pure fractions might be expected to be less stable than the original juice. This was actually found to be the case. An active fraction was prepared from decitrated lemon juice by removing inactive material by clearing the juice with neutral lead acetate at  $p_H$  5.4 and then precipitating the active principle by raising the  $p_H$  of the filtrate to 7 [Zilva, 1927, 1]<sup>1</sup>.

<sup>1</sup> Such preparations are usually active in doses equivalent to 1.5 cc. of the original juice in so far that the guinea-pigs survive on them the test period of 60 days. Occasionally such a degree of activity is not attained but the cause of this variation has not yet been ascertained.

Doses of this fraction equivalent to 5 cc. of the original juice were tested immediately after preparation, after 24 hours' and after 72 hours' storage in the cold room. Four guinea-pigs were used in each set. The animals to which the dose was administered immediately after preparation were chloroformed after 61 days, signs of mild scurvy being established at the post-mortem. Both sets of animals on the stored preparations succumbed to scurvy within a month, *i.e.* the stored doses showed no activity at all. The reducing capacity for phenolindophenol of the active fraction was about one-third of that of the original decitrated lemon juice from which it was prepared. In another experiment a fraction prepared as above was fed immediately after preparation and after 7 days' storage in doses equivalent to 1.5, 3 and 5 cc. of the original juice. In the former case the guinea-pigs survived for 60 days even on the lowest dose, whilst in the latter all the 9 animals, including those receiving the highest dose, succumbed to scurvy within a month. As ordinary decitrated lemon juice of similar antiscorbutic activity does not deteriorate so quickly one may reasonably infer that some substance or substances which have a protective action on the vitamin have been removed in the process of fractionation.

*The dialysing properties of the reducing agency.*

In view of the fact that, when decitrated lemon juice is dialysed for 3 days in a collodion thimble previously soaked in 92 % alcohol, it loses its antiscorbutic activity, whilst when a thimble which has been soaked in 88 % alcohol is used under the same circumstances comparatively little loss in activity takes place [Zilva and Miura, 1921; Connell and Zilva, 1924], it was of interest to study the behaviour of the reducing agency under these differential conditions of dialysis. Ordinary decitrated lemon juice was, therefore, dialysed as described before in "92 %" and in "83 %" collodion thimbles in the presence of succinic acid for 3 days. The dialysed juice, as well as a control juice kept in a test-tube in the dialysing tank, were tested by daily administration to guinea-pigs for their antiscorbutic activity, whilst the doses were titrated daily with phenolindophenol before being administered to the test animals. An "83 %" collodion thimble was used in the experiment because thimbles with an 88 % alcohol index made from this particular sample of collodion yielded a juice of low activity after dialysis. The juice dialysed in the "83 %" thimble showed some activity in 1.5 cc. doses and full protection for 55 days in the higher doses, as did also the control non-dialysed juice. The decitrated lemon juice dialysed in the "92 %" thimble, as was to be expected, was found to be inactive, even in 5 cc. doses. The phenolindophenol titrations revealed a total loss of the reducing agency in solutions dialysed in the "92 %" thimbles, an approximate loss of more than a half in the solutions dialysed in the "83 %" thimbles, and a loss of less than a quarter in the non-dialysed control. These observations suggest the necessity of revising the interpretation of the results in connection with the dialysing properties



of the antiscorbutic factor. It was previously assumed by the writer and his collaborators that because active solutions when dialysed through membranes with an alcohol index of 92-95 % became inactive, the vitamin had diffused out. Since, as we have seen from the above experiments, the reducing agency diffuses under these conditions, one cannot dismiss the possibility that the active molecule may be larger than was hitherto surmised and that by the diffusion of the reducing agency it becomes inactivated without actually diffusing out of the thimble. The matter requires further investigation.

*The effect of acidity on the stability of the antiscorbutic factor in anaerobically autoclaved decitrated lemon juice.*

In the previous communication it was shown that when decitrated lemon juice was autoclaved under strictly anaerobic conditions and stored, the antiscorbutic activity deteriorated quickly. The reaction of such solutions during storage was neutral. It was of interest to ascertain whether this deterioration on storage in the treated juice would also take place in an acid medium. The juice was, therefore, autoclaved as previously described, acidified to  $p_H$  3 by the addition of citric acid and stored in the cold room for 7 days, after which time it was tested on the guinea-pigs. Seven out of the nine animals survived the test period of 62 days, showing little scurvy at the autopsy. Of the other two guinea-pigs, one, receiving a 1.5 cc. dose, died after 44 days, and the other, receiving a 3 cc. dose, died after 59 days from an intercurrent disease<sup>1</sup>. The control, non-acidified juice was found, as usual, to be inactive in 3 cc. doses. Experiments with autoclaved preparations stored at  $p_H$  6 and at  $p_H$  6.7 showed that the deterioration on storage was *definitely* retarded even at these hydrogen ion concentrations.

*The effect of acidity during the process of autoclaving on the stability of the antiscorbutic factor and of the reducing agency in lemon juice.*

Although an acid reaction retards the deterioration of the antiscorbutic activity of autoclaved decitrated lemon juice, it does not prevent, during autoclaving, the change which conduces to the accelerated deterioration of the active juice on storage. This was demonstrated by two experiments. In the first experiment decitrated lemon juice was brought up to  $p_H$  6 by the addition of citric acid and autoclaved as before for 1 hour. After cooling it was adjusted to  $p_H$  7 and kept at this hydrogen ion concentration for 7 days in the cold room before being tested. This stored preparation was found to be inactive even in doses of 5 cc. and had lost almost entirely its capacity for reducing phenolindophenol. In the second experiment freshly expressed lemon juice was autoclaved under strictly anaerobic conditions in the usual way. It was then decitrated, adjusted to  $p_H$  7 and stored in this neutral condition in the cold room for 7 days before feeding. As a control, the autoclaved lemon

<sup>1</sup> These stored juices were found to reduce phenolindophenol but their reducing capacity was greatly diminished.

juice was tested *immediately* after decitration. Decitrated lemon juice autoclaved anaerobically at  $p_H$  7, and administered at once and after 7 days' storage at  $p_H$  7, was also tested at the same time. Although the guinea-pigs dosed on the autoclaved lemon juice immediately after decitration survived for 60 days even on the lowest dose of 1.5 cc., little activity was revealed by this juice when stored for 7 days at  $p_H$  7. There was a slight delay in the death of the guinea-pigs receiving the higher doses, but all the 9 animals in the set died of scurvy within 30-40 days. As usual, decitrated juice autoclaved at  $p_H$  7 and fed at once was found to be active, whilst after 7 days' storage the same juice failed entirely to protect in doses of 3 cc. The autoclaved lemon juice, immediately after decitration, reduced phenolindophenol almost to its full extent. On storage, this reducing capacity deteriorated to a very great extent, *i.e.* some of the stored specimens showed no reduction at all, in others, the titre was reduced to about a fifth or sixth of its original value.

*The effect of heating to 143° on the antiscorbutic factor in decitrated lemon juice.*

In this experiment decitrated lemon juice at  $p_H$  7 was alternately evacuated and washed out with oxygen-free nitrogen three times and eventually autoclaved for 1 hour at 10 lbs. pressure (113°) in the evacuated ampoule. After cooling the heated juice was tested at once. Of the guinea-pigs on the 1.5 cc. doses, one died after 14 days, another after 17 days and the third was chloroformed after 61 days. All the animals on this dose showed signs of scurvy at the post-mortem examination. Two guinea-pigs on the 3 cc. dose were chloroformed at the end of the test period of 61 days and were found to be free from scurvy. The third animal died of an intercurrent disease after 60 days, showing mild signs of scurvy. A similar result was obtained with the highest dose of 5 cc. Two of the animals were *fully* protected from scurvy for 61 days after which time they were chloroformed, the third animal died of an intercurrent disease after 60 days showing some signs of scurvy at the autopsy. It is, therefore, seen that, in spite of the very drastic treatment, the loss in the antiscorbutic potency of the juice was not great—a loss most probably due not to the thermal degradation of the vitamin but to the presence of very slight traces of oxygen or to other slight imperfections in the technique.

#### CONCLUSIONS.

The experiments dealt with in this communication are recorded with the object of lending further support to the suggestion made in the earlier paper previously quoted that the stability of the antiscorbutic factor in lemon juice is conditioned by the presence of a reducing principle and of a factor, the functioning of which is destroyed by heat. It was then suggested that the reducing property of the solution acted as a "reduction buffer" for the antiscorbutic vitamin. The results obtained in this investigation seem to justify this hypothesis. It is seen that after the reducing properties have been

destroyed the antiscorbutic activity does indeed disappear very much more rapidly. The dependence of the reducing agency, and consequently of the antiscorbutic factor, on the heat-labile factor is confirmed. Although acidity does not prevent the destruction of this last factor by heat, it exercises definite protection of the reducing and of the antiscorbutic principles after anaerobic heating. In fact, the change effected by autoclaving is such that the heated active solution becomes almost as unstable in the neutral zone as an unheated active solution is in the alkaline region—a change which is most probably due to an increased susceptibility to oxidation. We are thus faced with a vague picture of a complex mechanism in which the following points stand out in relief. The antiscorbutic factor, a principle which can withstand such drastic treatment as heating at  $143^{\circ}$  in the absence of air, becomes very labile in the presence of air, on changing the protective conditions of its natural medium. Experiments on purified fractions reveal that the process of chemical purification is one of the means of removing this protection.

As a mode of expression the writer has been compelled to employ such terms as “reducing agency” and “thermo-labile factor.” This needs some qualification. The experimental evidence so far produced does not warrant the assumption of the existence of such substances in a preformed state in the cell and consequently the possibility of the presence of a large active molecule which, on destruction of the tissues of the plant, is degraded or modified and thus rendered less stable, must be borne in mind. This can only be decided by further work.

#### SUMMARY.

When phenolindophenol is added to decitrated lemon juice until the indicator is no longer reduced and the solution is adjusted immediately to  $p_H$  7, the antiscorbutic activity disappears within 24 hours.

Purified antiscorbutic fractions from lemon juice lose their activity much more rapidly than does decitrated lemon juice of similar activity.

Decitrated lemon juice dialysed in collodion thimbles of a permeability which leaves the solution inactive after 3 days, loses the capacity for reducing phenolindophenol. This reducing capacity is retained to a great extent by the juice when dialysed in thimbles of a permeability which yields an active juice at the end of the dialysis.

Acidity retards the deterioration, on storage, of the antiscorbutic activity in anaerobically autoclaved decitrated lemon juice. On storage at  $p_H$  3 the deteriorating effect of autoclaving is hardly perceptible.

Lemon juice autoclaved anaerobically, even in a very acid medium, deteriorates much more rapidly at  $p_H$  7 on storage than similar solutions which have not been autoclaved.

Comparatively little loss is registered in decitrated lemon juice which has been autoclaved at 40 lbs. pressure ( $143^{\circ}$ ) for 1 hour under *strictly* anaerobic conditions.

## REFERENCES.

- Connell and Zilva (1924). *Biochem. J.* 18, 632.  
Zilva (1923). *Biochem. J.* 17, 410.  
—— (1927, 1). *Biochem. J.* 21, 354.  
—— (1927, 2). *Biochem. J.* 21, 689.  
Zilva and Miura (1921). *Biochem. J.* 15, 422.

## C. FERMENTATION BY DRIED YEAST PREPARATIONS. II.

BY ARTHUR HARDEN AND MARJORIE GIFFEN MACFARLANE.

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*(Received May 2nd, 1928.)*

It has been shown [Harden, 1925] that the addition of sodium acetate or bicarbonate accelerates the onset of fermentation by zymine in a large volume of sugar solution. Further investigation shows that this acceleration is produced by the presence of other salts, both organic and inorganic, the induction time varying with the concentration and with the specific nature of the salt.

To avoid irregularities previously noticed, the fermentations were always carried out in the following manner: 0.2 g. zymine was wetted for 1 minute with 1 cc. water; to this were added (1) the salt solution, (2) 0.5 cc. 20 % fructose, and (3) water to make a total volume of 2.5 cc. The mixture was saturated with  $\text{CO}_2$  and incubated at  $29.5^\circ$ . A period of 5 minutes was allowed for the attainment of an even temperature, after which readings were taken at 5 minute intervals. In each series, a control flask containing zymine, sugar and water was incubated to give the normal induction time for this volume of sugar solution; this varied from 40 to 60 minutes according to the sample of zymine. For the purpose of comparison of the effects of various salts, the induction period has been fixed arbitrarily as the interval of time between the mixing and the attainment of a rate of fermentation of 0.2 cc. in 5 minutes.

The initial  $p_{\text{H}}$  of the mixtures after saturation with  $\text{CO}_2$  was determined by the capillator method in duplicate flasks; the final  $p_{\text{H}}$  was also determined in the earlier experiments. No difference in the initial  $p_{\text{H}}$  on the addition of the salts could be detected, while that between the initial and final  $p_{\text{H}}$  was never more than 0.2, the initial  $p_{\text{H}}$  being 5.6.

The salts tested and the length of the induction period at the optimum concentrations found are given in Table I. With the exception of sodium arsenate, all the salts tested reduce the induction period materially, the optimum concentration, at least with the inorganic salts, appearing to be dependent on the nature of the anion. Thus the optimum varies from 0.1  $M$  in the case of chlorides to 0.02  $M$  for sulphates and phosphates and 0.002  $M$  for sodium citrate. The last is peculiar in showing a sharply marked optimum concentration, for the other organic salts employed were effective within wide limits of concentration, and with less distinction between uni- and bi-valent anions.

Table I.

Salt	Optimum concentration (molar)	Minimum induction time (min.)
— ...	—	60
KCl ...	0.05–0.1	20
NaCl ...	0.1	25
NH <sub>4</sub> Cl ...	0.05–0.1	20
K <sub>2</sub> SO <sub>4</sub> ...	0.01–0.05	20
Na <sub>2</sub> SO <sub>4</sub> ...	0.01–0.02	20
MgSO <sub>4</sub> ...	0.01	20
Na <sub>2</sub> HPO <sub>4</sub> ...	0.02–0.04	20
K formate ...	0.2	25
K acetate ...	0.2	15
K propionate ...	0.05–0.4	10
K lactate ...	0.2	15
K succinate ...	0.05–0.1	20
K tartrate ...	0.05–0.1	20
Na citrate ...	0.002	20
Na glycerophosphate ...	0.04	20
Na hexosediphosphate ...	0.011 (minimum)	10
Na trehalosemonophosphate	0.016 (minimum)	15

Of the organic salts, potassium formate, tartrate, succinate, lactate and citrate have approximately the same effect as the inorganic salts; that is to say, the induction time is reduced from 60 to 20 minutes in their presence. Potassium propionate and acetate rank with the sodium salts of hexose-diphosphate and trehalosemonophosphate in producing an immediate fermentation. The effective concentration of K propionate covers a wide range, 0.05 to 0.4 *M*, but one whose limits have been definitely ascertained. In the case of the hexosephosphoric esters, a minimum concentration of 0.01 *M* is necessary for the immediate onset of fermentation, but it has not yet been possible to determine whether a high concentration will eventually increase the induction time as is the case with other salts.

The position of sodium arsenate (Table II) with respect to this effect of salts is unique, in that it acts in a direction contrary to that of all the other salts tested. In concentrations as low as 0.00005 and 0.0002 *M*, the induction time is normal or slightly increased; as the concentration is raised, the induction time lengthens. This effect is all the more striking as, when fermentation does begin, the rate of fermentation is greatly increased above the normal, as is usual in the presence of arsenate. The length of the induction time has no deleterious effect upon the subsequent fermentation. This is again in contrast with the action of high concentrations of inorganic salts by which the induction time is prolonged and the subsequent rate of fermentation is diminished [Harden and Henley, 1921]. Furthermore, in the presence of arsenate, the beneficial effect of added salts, both organic and inorganic, is partially counteracted. Thus, in the presence of 0.1 *M* sodium chloride and 0.002 *M* arsenate, the induction time is 50 minutes, whereas that with sodium chloride alone is 20 minutes and with arsenate alone, 75 minutes. These results are shown in Table II.

Table II.

Induction time (min.) for 0.2 g. zymin in 2.5 cc. 4 % sugar solution in presence of arsenate ions and added salts.

Concentration of $\text{Na}_2\text{HAsO}_4$ (molar)	$\text{H}_2\text{O}$	0.1 <i>M</i> KCl	0.2 <i>M</i> Na acetate	0.002 <i>M</i> Na hexose- diphosphate
0	60	10	15	20
0.0002	65	20	—	—
0.002	75	45	45	55
0.01	120	—	—	—

The action of sodium arsenate in prolonging the induction time without any toxic effect on the ensuing fermentation appears to be specific; it is difficult, however, to link this with the only known effect of arsenate ions in the fermentation process, viz. an increase in the rate of decomposition of hexosediphosphate, more especially as the beneficial action of this salt is counteracted equally with that of the inorganic salts by the presence of arsenate in the mixture. It is possible, however, that the action of hexosediphosphate and trehalosemonophosphate is in no way specific but falls in with the general effect of salts.

The phenomenon of induction under discussion presents some analogy to the induction which occurs in maceration extract and which has been studied by Meyerhof [1918]. In the latter case, however, the period of induction is completely abolished by the presence of hexosediphosphate in as small a concentration as 0.0002 *M*. It is moreover greatly diminished by the substitution of sucrose for glucose and fructose, which is not the case with the delayed fermentation of zymin. Meyerhof also found that the induction was removed by grinding the dried yeast with glass powder before preparing the maceration extract; grinding the zymin with glass powder, however, produced no effect on the induction period in the present experiments (see Table III).

Table III.

Induction time (min.) for 0.2 g. zymin in 2.5 cc. 4 % sugar solution.

Variation in conditions	Sugar		
	Fructose	Glucose	Sucrose
0.2 g. zymin unground ... ..	60	55	60
0.2 g. zymin + 0.1 g. glass powder ... ..	60	—	—
0.2 g. zymin ground with 0.1 g. glass powder ... ..	60	—	—
0.2 g. zymin + 0.2 cc. 1 % aldehyde... ..	50	—	—

The "induction" period in the case of zymin placed in a relatively large volume of sugar solution also appears to correspond to some extent with the period which elapses before the attainment of the maximum rate (Meyerhof's "Gäranstieg") in the fermentation of sugars in presence of phosphate by yeast preparations. Unlike this, however, it is very little affected by the addition of aldehyde (see Table III) so that it is probably not due to the same cause.

The results so far obtained render it probable that the induction period under discussion is due to the slow accumulation in the solution of one of the factors necessary for rapid fermentation. Whether this is accomplished by direct extraction from the zymin, a process which is aided by the presence of salts, or whether the process is a more complicated one, involving both the extraction and some subsequent chemical change before fermentation can proceed rapidly, remains to be ascertained.

#### SUMMARY.

1. It has been found that by adding various inorganic and organic salts to zymin placed in a large volume of sugar solution, the period of induction normally occurring before the onset of rapid fermentation is appreciably reduced.

2. Sodium arsenate is an exception to this general salt effect in that it prolongs the induction period without toxic effect on the fermentation.

#### REFERENCES.

- Harden (1925). *Biochem. J.* **19**, 447.  
Harden and Henley (1921). *Biochem. J.* **15**, 312.  
Meyerhof (1918). *Z. physiol. Chem.* **102**, 183.



## CI. THE DUAL NATURE OF WATER-SOLUBLE VITAMIN B. II.

### THE EFFECT UPON YOUNG RATS OF VITAMIN B<sub>2</sub> DEFICIENCY AND A METHOD FOR THE BIOLOGICAL ASSAY OF VITAMIN B<sub>2</sub>.

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*(Received May 3rd, 1928.)*

IN a previous communication [Chick and Roscoe, 1927], experiments were described which confirmed the conclusion of Goldberger and his colleagues [1925, 1926] that the water-soluble B vitamin as defined by the discoverers, McCollum and Davis [1915, 1, 2], had two components<sup>1</sup>.

(1) Vitamin B<sub>1</sub> or the antineuritic, less heat-stable vitamin discovered by Eijkman in 1897, deficiency of which in diet leads to polyneuritis and death in birds, and to death, with or without paralysis, in rats and other mammals.

(2) Vitamin B<sub>2</sub>, a more heat-stable vitamin, often accompanying vitamin B<sub>1</sub> in natural foodstuffs, in the absence of which the animal fails to grow and, in the case of rats, suffers from dermatitis.

Deficiency of vitamin B<sub>1</sub> is usually held to be the cause of beriberi in man, and, according to Goldberger and his colleagues, deficiency of vitamin B<sub>2</sub> is the cause of human pellagra.

When young rats are placed upon an experimental diet complete in all other respects, they show no growth if *either* of these two vitamins is lacking. In absence of vitamin B<sub>1</sub>, death usually occurs in 3–4 weeks; in absence of B<sub>2</sub>, the rats survive much longer. There is, therefore, no justification for calling vitamin B<sub>2</sub> a "growth factor" in contradistinction to vitamin B<sub>1</sub>, as has been done by some writers.

That the water-soluble B vitamin and the antineuritic vitamin were not identical, as was supposed by McCollum and Kennedy [1916] and others, has long been suspected and a résumé of the literature on the subject was given in our previous paper [Chick and Roscoe, 1927]. Since the work of Goldberger and his colleagues was published, however, many papers besides our own have appeared which bear upon this point. In some, the separate action of vitamins B<sub>1</sub> and B<sub>2</sub> is clearly demonstrated, in others, it can be inferred.

<sup>1</sup> The nomenclature here used is in accordance with that provisionally adopted by the Biochemical Society.

Smith and Hendrick [1926] found that a synthetic diet devoid of water-soluble B vitamins was not rendered adequate to maintain growth in rats by adding Seidell's antineuritic concentrate (picrate) prepared from yeast, but required to be supplemented by an additional dietary factor. This second factor was also contained in yeast, and was able to withstand heating in the autoclave at 15 lbs. pressure for 6 hours.

Hauge and Carrick [1926], from observations on the growth and development of baby chicks, concluded that yeast contained a water-soluble vitamin necessary for growth which was separate from the antineuritic factor.

Salmon [1927] found that the seeds of the velvet bean were more potent than the leaves in preventing polyneuritis in pigeons fed on polished rice, but that the reverse was true for maintenance of growth in young rats when one or other of these two products was used to supplement experimental diets devoid of water-soluble B vitamins. Salmon succeeded in making a partial separation of the two water-soluble vitamins from the leaves of the velvet bean by adsorbing the antineuritic vitamin on fuller's earth, but in order to maintain growth in rats this antineuritic solid required to be supplemented with the filtrate yielded after treatment with the earth. In a later paper Salmon, Guerrant and Hays [1928] reported a more complete separation of these two principles to which they gave the names vitamin P-P, as suggested by Goldberger (= B<sub>2</sub>) and B-P (= B<sub>1</sub>). They found (1) that vitamin B<sub>1</sub> was adsorbed without appreciable admixture of vitamin B<sub>2</sub> if the extract made from the leaves were treated with a very small quantity of fuller's earth (10 g. per kg. air-dried leaves), and (2) that vitamin B<sub>2</sub> was precipitated with very small admixture of vitamin B<sub>1</sub>, if alcohol to 82.7 % (by weight) were added to the leaf extract, after the latter had been previously treated several times with fuller's earth to remove vitamin B<sub>1</sub>.

Williams and Waterman [1927], also using fuller's earth, removed a substance from an aqueous extract of yeast which was antineuritic but unable to maintain growth of rats on diets devoid of water-soluble B unless supplemented, for example, by yeast which had been heated for 6 hours at 125°.

Sherman and Axtmayer [1927] found that a supplementary relationship existed between whole wheat and autoclaved yeast and between whole wheat and dried skimmed milk when these were used to supply "vitamin B" to an otherwise complete diet. From these results the authors deduce the existence of two separate dietary factors in "vitamin B," both necessary for growth, for which they propose the names vitamin F (antineuritic) and vitamin G (heat stable).

In a series of recent papers Palmer and Kennedy [1927, 1, 2; 1928], have described the difficulties encountered in designing a "synthetic" diet, prepared from highly purified foodstuffs, which should be entirely satisfactory for growth and reproduction of rats. "Vitamin B" was supplied by an alcoholic extract of wheat embryo and highly purified caseinogen was the source of protein. The nutritive value was much improved when the diet was supple-

mented by autoclaved yeast or when commercial caseinogen was substituted for the highly purified product (see below, p. 793). It seems probable, though not proved, that vitamin B<sub>2</sub> is the missing constituent in their apparently complete "ration 5." This conclusion agrees with the results of recent investigations by Evans and Burr [1928] on the difference in growth of rats maintained on rations containing, respectively, purified caseinogen and sugar and the commercial products. They conclude that vitamin B<sub>2</sub> is present in the latter but not in the former. They also find that "Tikitiki," the dilute alcoholic extract of white rice polishings distributed to the natives by the Philippine Bureau of Science, possesses the antineuritic vitamin while "almost entirely lacking the growth-promoting vitamin B."

The technique which enabled us to demonstrate the separate effect on nutrition of these two vitamins was the use of the concentrated antineuritic vitamin prepared from yeast by the method of Peters [Peters, 1924; Kinnersley and Peters, 1925]. This product contains vitamin B<sub>1</sub> without any admixture of vitamin B<sub>2</sub> [Chick and Roscoe, 1927]. As a source of vitamin B<sub>2</sub>, free from B<sub>1</sub>, we used whole yeast autoclaved for 5 hours at 120°. This degree of heating destroys all the antineuritic vitamin contained in yeast, but only about one-half of the vitamin B<sub>2</sub> originally present.

Since our earlier paper was written some improvements in animal technique have been devised which have enabled us to make a more accurate study of the effect of vitamin B<sub>2</sub> deficiency on young growing rats. Incidentally, a method has been evolved for evaluation of vitamin B<sub>2</sub> which appears to possess a reasonable degree of accuracy for a biological method.

*Improvements in animal technique required for study of vitamin B<sub>2</sub>.*

In our earlier experiments [Chick and Roscoe, 1927] we found that young rats on diet L<sup>1</sup> (devoid of water-soluble vitamins) and receiving vitamin B<sub>1</sub>, in the form of a small daily ration of Peters's antineuritic concentrate, failed to grow and within 7-10 weeks developed the dermatitis and skin lesions described by Goldberger and Lillie [1926]. In subsequent experiments, however, skin symptoms were absent, although the animals were maintained for long periods on the diet and no significant increase in weight occurred. Other workers, e.g. Macy, Outhouse, Long and Graham [1927], have had the same experience.

By analogy with observations made upon other vitamins, it seemed possible that the irregularity might be due to variations in the reserves of vitamin B<sub>2</sub>.

<sup>1</sup> Diet L, devoid of water-soluble B vitamins:

Purified caseinogen	...	...	...	...	...	...	...	100 g.
Rice starch	...	...	...	...	...	...	...	300 g.
Cotton seed oil	...	...	...	...	...	...	...	75 g.
Salt mixture (McCormick's No. 185)	...	...	...	...	...	...	...	25 g.
Water	...	...	...	...	...	...	...	500 cc.
Cod-liver oil given daily by hand 0.05-0.1 g., according to the size of the rat.								

The diet is heated, before use, for 3 hours in steam at 100°, in order to prevent the possibility of "refection" in the experimental animals [see Roscoe, 1927].

held by the animals. Although the young rat is incapable of storing any considerable quantity of vitamin B<sub>1</sub>, the long period of survival when deprived of vitamin B<sub>2</sub> suggested a capacity for its storage.

Attempts were, therefore, made to reduce the supply of water-soluble B vitamins during lactation by limiting the mother's diet to rice, polenta (maize endosperm), white bread and milk, marmite being omitted. In case of one litter thus bred, 2 out of 3 receiving the "— B<sub>2</sub>" diet developed the typical skin appearances; of these, one (No. 212) died, but the other (No. 213) showed some degree of spontaneous recovery. In the case of another litter, two rats (Nos. 172 and 178) survived more than 20 weeks without developing any skin lesions. These results showed no improvement in consistency and it was necessary to look elsewhere for the causes of the irregularity.

We next considered the possibility that our basal diet might not be sufficiently free from vitamin B<sub>2</sub>. We had hitherto used caseinogen prepared commercially from skim milk by precipitation with acid, washing with acidulated water, drying and prolonged heating at 120°. The caseinogen was not extracted with alcohol, so that, while the heating to which it was subjected might have destroyed vitamin B<sub>1</sub>, vitamin B<sub>2</sub> might still be present, and our irregular results might be due to the use of batches of caseinogen containing different amounts of B<sub>2</sub>.

We therefore purified caseinogen as follows. "Light white soluble" caseinogen was dissolved to form a 5 % solution, reprecipitated with acetic acid and washed by decantation with 0.05 % acetic acid, the supernatant fluid being changed twice a day for a fortnight. The caseinogen was then extracted with dilute acidified alcohol (50–70 % strength (by volume), containing about 0.03 % acetic acid) for 96 hours in a large Soxhlet apparatus, and afterwards washed with 93 % alcohol, dried in an electric oven at a low temperature, ground in a mill and finally roasted for 3 days at 120°.

The behaviour of young rats 40–50 g. in weight receiving the "— B<sub>2</sub>" diet prepared with this highly purified caseinogen was compared with that of others from the same litter receiving a similar diet made with the caseinogen previously used. After 12 weeks the difference between the two sets of rats was striking. Two rats receiving the commercially purified caseinogen had poor coats and had shown no significant increase in weight (weight 52 g.) but they were in fair condition, they showed no skin symptoms, and their eyes remained normal (see rats 218 and 222, Fig. 1). The weight of 7 rats receiving the specially purified caseinogen ranged from 39 to 47 g., 5 showed typical skin symptoms, all had sunken eyes and inflamed eyelids and their general condition was miserable (see rats 217, 220 and 221, Fig. 1).

It is evident, therefore, that vitamin B<sub>2</sub> is present in precipitated caseinogen, and can be removed by thorough extraction with acid water and acid alcohol. This is a point of some importance and, in part at least, may account for the high nutritive value possessed by caseinogen as a protein. It probably explains the improvement observed by Palmer and Kennedy [1927, 2] in the nutri-

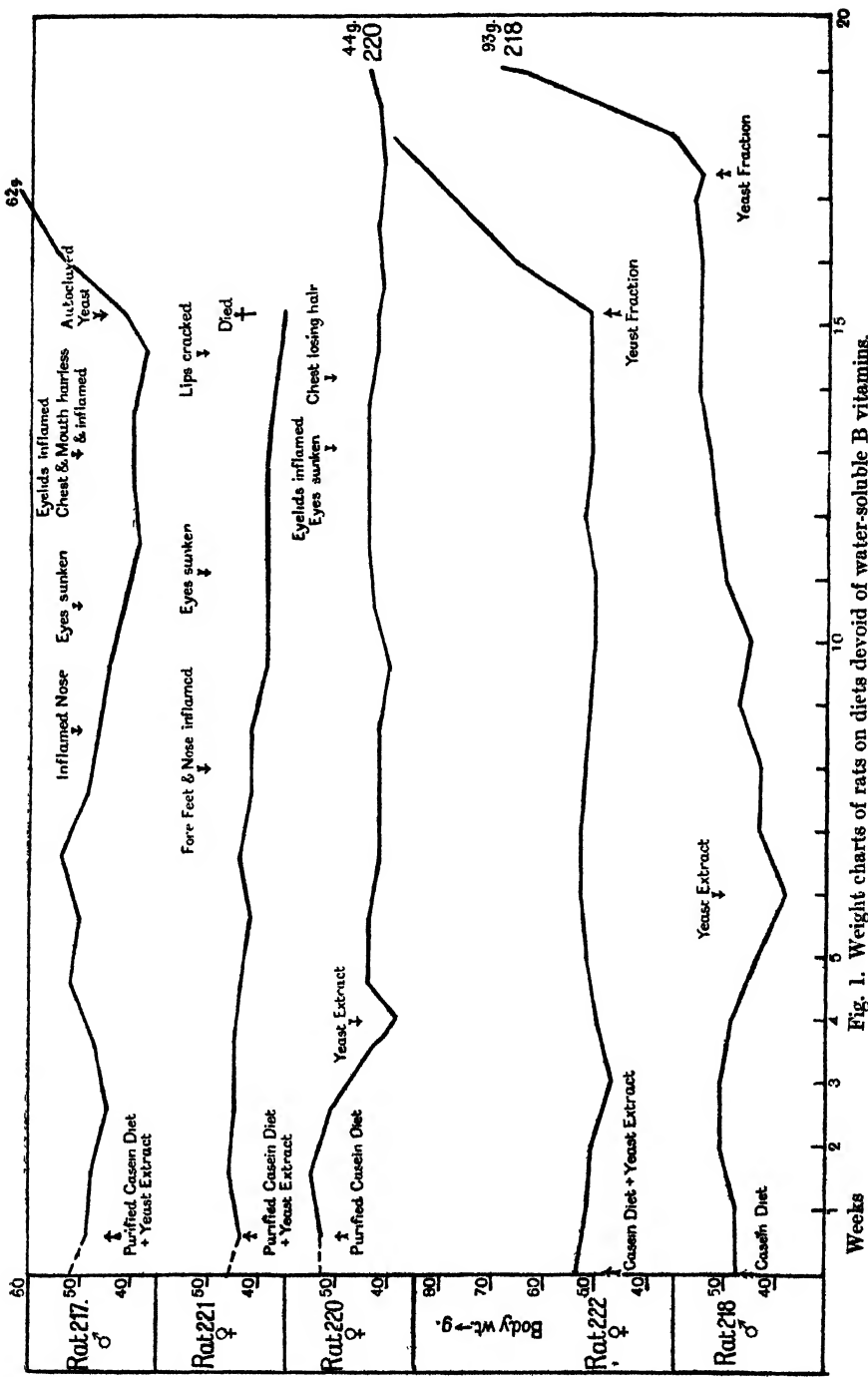


Fig. 1. Weight charts of rats on diets devoid of water-soluble B vitamins.  
Yeast extract contains B<sub>1</sub>.  
Yeast fraction contains B<sub>6</sub>.

tional value of their "synthetic" ration for rats, when a commercial preparation of caseinogen was substituted for their highly purified product. The disappointing results obtained by Goldberger and Tanner [1925] with highly purified caseinogen, as compared with milk and milk products, when these were added to the diet of inmates in Institutions where pellagra was endemic, may possibly have a similar explanation.

*Symptoms shown by young rats on a diet deprived of vitamin B<sub>2</sub>.*

Young rats can often be maintained for 3 months and longer upon a diet deprived of vitamin B<sub>2</sub>. During this period they will show no significant increase or loss in weight (see Fig. 1). They do not, however, appear to be ill or distressed, they have a fair appetite and remain active and interested in their environment. The skin symptoms, which are symmetrically placed on the animal, gradually develop at any time after the 5th or 6th week, but do not usually become severe until several weeks later. The most constant symptoms in our experience are the following. (1) Dermatitis and loss of hair from the eyelids, which may become stuck together; if the eyelids are loosened by bathing with warm water, the eyes, though much sunken, appear to be healthy. (2) Front paws stained with blood, caused by rubbing the inflamed margins of the nostrils; wetting of the lower portion of the abdomen with blood stained urine. (3) Dermatitis and loss of fur on head, round the nose and mouth and on the abdomen. Inflammation of the skin at the tips of the ears and a curious and very characteristic oedematous dermatitis of the digits of the paws, which become bright red in colour, were constant early symptoms in our earlier experiments, but at present are encountered later and much less frequently.

The irregularity in the occurrence of the various skin symptoms and the occasional instances of animals deprived of vitamin B<sub>2</sub> remaining stunted in growth but exhibiting no special skin lesions, suggest that we may possibly be dealing with more than one kind of dietary deficiency. There has certainly

Fig. 1. Weight charts of 5 young rats from the same litter on basal diets devoid of water-soluble B vitamins. Vitamin B<sub>1</sub> given as Peters's concentrated antineuritic extract from yeast (daily dose = 0.6 g. dry yeast). Rats 217, 220, 221 and rats 218, 222, received diets prepared from the more and less highly purified caseinogen respectively.

*Rat 217* (initial weight, 52 g.) received vitamin B<sub>1</sub> throughout, developed typical skin lesions after 9 weeks; at 15 weeks (weight 39 g.) received vitamin B<sub>2</sub> as 0.4 g. daily of yeast autoclaved for 5 hours at 120°.

*Rat 221* (initial weight, 46 g.) treated similarly to rat 217, developed skin lesions after 8 weeks, remained untreated and died after 15 weeks of vitamin B<sub>2</sub> deficiency (weight 35 g.).

*Rat 220* (initial weight 53 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B<sub>1</sub> at 4½ weeks, was restored by administration of vitamin B<sub>1</sub>, developed skin lesions after the 13th week (weight after 19 weeks, 44 g.).

*Rat 222* (initial weight 54 g.) received vitamin B<sub>1</sub> throughout; at 15 weeks had developed no skin symptoms (weight 51 g.); administration of vitamin B<sub>1</sub> in the form of a yeast fraction, caused increase in weight of 39 g. in 3 weeks.

*Rat 218* (initial weight 48 g.) at first deprived of both B vitamins, showed collapse (weight 38 g.) due to lack of vitamin B<sub>1</sub> at 6 weeks, restored by administration of vitamin B<sub>1</sub>, showed no skin symptoms in 18 weeks (weight 54 g.); administration of vitamin B<sub>2</sub>, in form of a yeast fraction, caused immediate increase in weight (46 g. in 2 weeks).

been greater constancy in the development of symptoms since we have taken steps to free the caseinogen in the basal diet from possible contamination with water- or alcohol-soluble vitamins, but, on the other hand, the typical dermatitis of the paws and ears, for example, which is now so infrequent, was previously often observed in rats upon diets prepared with less pure caseinogen and receiving a small, though insufficient, supply of vitamin B<sub>2</sub> in the form of autoclaved yeast [see Chick and Roscoe, 1927, Table II].

Our experience also differs in some respects from that of Findlay [1928] who records "moderate growth...for from 4 to 6 weeks" in rats deprived of vitamin B<sub>2</sub>, followed by "slight but gradual loss of weight" with development of skin lesions. We have not observed the change in temper of the animals, those previously tame and docile becoming irritable and liable to bite, described by him, nor have we observed any great loss in appetite. In our experience, the intake of food remains rather high (about 4-6 g. daily dry weight) seeing that the body weight of the animals remains steady at about 40-50 g.

*Recovery on addition of vitamin B<sub>2</sub> to the diet.*

If the condition of the rats after many weeks on the "— B<sub>2</sub>" diet is not too bad, they respond promptly to administration of vitamin B<sub>2</sub> and increase in weight begins immediately, often within 24 hours. If the supply of vitamin B<sub>2</sub> is abundant the growth in weight may be far in excess of the normal for a time and we have frequently observed gains of 20-30 g. a week for a short period (see Table I and Curves of rat 218, Fig. 1). The sunken eyes are usually the next to show improvement and soon begin to protrude. Desquamation of the affected skin also takes place, after which the fur begins to grow and the animal gains a normal appearance within 2-4 weeks, according to the severity of the previous symptoms. It remains for some time undersized for its age.

*Method for evaluation of vitamin B<sub>2</sub>.*

If the vitamin B<sub>2</sub> is supplied after only a few weeks of deprivation and before the animal's condition is too bad, the response in growth is at first roughly in proportion to the amount of vitamin B<sub>2</sub> administered. It is well, however, not to leave the animal too long on the deficient diet (see rat 251, Table I) and there is no need to await the development of specific symptoms.

Young rats are placed upon the basal diet, deprived of B vitamins, when about 4 weeks old and 40-50 g. in body weight. They are left together in a large cage for about 10 days. After this time, they are placed in separate cages, because of their tendency to pull out and eat the fur of their companions. The cages have floors of wide-meshed ( $\frac{1}{8}$ -inch) zinc gauze, raised about 2 inches above trays filled with peat moss litter. This precaution is necessary in order to hinder the consumption of the faeces, which is a common practice of rats on diets deprived of B vitamins, and tends to obviate the effect of the dietary deficiency [Steenbock, Sell and Nelson, 1923].

Table I. *Titration of vitamin B<sub>2</sub> by increase of body weight in young rats, which have ceased to grow on "– B<sub>2</sub>" diet, i.e. diet L, devoid of B vitamins, to which vitamin B<sub>1</sub> is added as Kinnersley and Peters's concentrate from yeast.*

Material tested	Daily dose expressed as the equivalent of dry yeast g.	Rat No.	Body weight when dose was started g.	Weekly growth increments while receiving dose g.	Mean for rats receiving a given dose g.	Time previously maintained on "– B <sub>2</sub> " diet Weeks
Fraction 5 I, extract of Yeast V in 0.01 % acetic acid evaporated to small bulk	1.2	142	116	36, 32	} 26 24	14
	1.2	140	101	24, 12		14
	0.9	243	45	26, 22		3
Fraction 5 II, prepared as 5 I, from Yeast V and VII	0.5	254	41	21	} 21	2
	0.5	255	41	22		2
	0.5	256	43	19		2
	0.25	254	71	14, 11	} 11	} 2 + 2 on above test + 2 deprived until weight was again constant
	0.25	255	65	14, 12		
	0.25	256	66	8, 11		
Fraction 5 III, prepared as 5 I, from Yeast VIII	1.0	251	45	24, 28	26	15 + 1 receiving the equivalent of 0.5 g. yeast with no response
	0.5	251	43	2	—	15
	0.5	268	52	20, 15.5	18	6
	0.25	272	53	10.5, 13.5	} 11	7
	0.25	279	38	16, 17		4
	0.25	280	39	11, 11		2
	0.25	273	56	11, 9, 5, 9.5		2 + 2 receiving the equivalent of 0.12 g. yeast
	0.12	273	46	7, 3	} 7	2
	0.12	290	37	9, 12, 3		2
Dried Yeast	0.4	—	—	—	23	} See Chick and Roscoe [1927], Table III
	0.2	—	—	—	11	

The daily dose of Peters's antineuritic concentrate is omitted at first, for reasons of economy, but after about 2 weeks, it is given. A daily dose of 0.1 cc. (equivalent to 0.6 g. of the original yeast, dry weight) provides an excess of vitamin B<sub>1</sub>. It is necessary to remove all traces of lead and mercury from the preparation, seeing that it is to be fed regularly over long periods. This is conveniently done after the precipitation with Hopkins's reagent (acid mercuric sulphate) and before adsorption on norite charcoal. If precipitation with baryta is inserted after treatment with lead acetate, as recommended recently by Kinnersley and Peters [1927], the separation of the metallic sulphides is much facilitated.

The weight of the rat sometimes showed a temporary increase of a few grams after this inclusion of vitamin B<sub>1</sub> in the diet. After 3–4 weeks from the beginning of the experimental feeding, however, the young rat is prepared for testing the content of vitamin B<sub>2</sub> in any material which may be then administered. The minimum dose which gives an average weekly increase of 10–12 g. provides a convenient standard for comparison. It is not necessary



for the material tested to be freed from vitamin  $B_1$ , of which an excess is already present in the rat's diet.

Table I shows the results of titrating the vitamin  $B_2$  content of a yeast extract made with 0.01 % acetic acid, the first filtrate obtained in the Kinnersley and Peters process for preparing an antineuritic concentrate from yeast. The tests were made on extracts from three different samples of yeast and show concordant results as long as the animals used have not been longer than 6-7 weeks on the deficient diet (see rat 251). For example, rat 268, which had been 6 weeks on the diet devoid of vitamin  $B_2$ , responded to a daily dose of Fraction 5 III, equivalent to 0.5 g. dry yeast, by an average increase in weight of 18 g. a week, whereas rat 251, after 15 weeks on the deficient diet, made no significant response to the same dose. This animal, however, grew 26 g. a week when it received the equivalent of 1 g. dry yeast. From the results obtained with rat 273 when testing Fraction 5 III, it appears justifiable to use the same rat successively for assay of two different doses of vitamin  $B_2$ , if the smaller dose is given first. If a larger is given first, an interval of 2 weeks without any dose should intervene before the smaller one is tested (see results of tests made with rats 254, 255 and 256 on Fraction 5 II).

The results obtained with Fractions 5 I, 5 II and 5 III, compared with those obtained with dried yeast, show that little loss of vitamin  $B_2$  has taken place during the extraction.

#### SUMMARY.

1. Additional evidence is given proving the existence of the two separate vitamins  $B_1$  and  $B_2$  in the "water-soluble B" vitamin complex (for definitions see p. 790).

2. An improved animal technique is described for the study of vitamin  $B_2$ , the most important modification being the use of highly purified caseinogen as source of protein in the basal diet. The antineuritic concentrate prepared by the method of Kinnersley and Peters is used as source of vitamin  $B_1$ .

3. A description is given of the effects observed in growing rats when they are fed on diets deficient only in vitamin  $B_2$ .

4. A method is described for assay of vitamin  $B_2$ .

5. It is suggested that the high value of caseinogen as a protein may be partly due to contamination with vitamin  $B_2$  and that the accepted nutritional value for this and other proteins may need revision.

Our thanks are due to Sir Charles Martin for his constant advice and criticism.

## REFERENCES.

- Chick and Roscoe (1927). *Biochem. J.* **21**, 698.  
Evans and Burr (1928). *J. Biol. Chem.* **77**, 231.  
Findlay (1928). *J. Path. Bact.* **31**, 353.  
Goldberger and Lillie (1926). *U.S. Treasury Pub. Health Rep.* **41**, 1025.  
Goldberger, Wheeler, Lillie and Rogers (1926). *U.S. Treasury Pub. Health Rep.* **41**, 297.  
Goldberger and Tanner (1925). *U.S. Treasury Pub. Health Rep.* **40**, 54.  
Hauge and Carrick (1926). *J. Biol. Chem.* **69**, 404.  
Macy, Outhouse, Long and Graham (1927). *J. Biol. Chem.* **73**, 153.  
McCollum and Davis (1915, 1). *J. Biol. Chem.* **23**, 181.  
——— (1915, 2). *J. Biol. Chem.* **23**, 231.  
McCollum and Kennedy (1916). *J. Biol. Chem.* **24**, 491.  
Palmer and Kennedy (1927, 1). *J. Biol. Chem.* **74**, 591.  
——— (1927, 2). *J. Biol. Chem.* **75**, 619.  
Peters (1924). *Biochem. J.* **18**, 858.  
Kinnnersley and Peters (1925). *Biochem. J.* **19**, 820.  
——— (1927). *Biochem. J.* **21**, 777.  
Kennedy and Palmer (1928). *J. Biol. Chem.* **76**, 591.  
Roscoe (1927). *J. Hyg.* **27**, 103.  
Salmon (1927). *J. Biol. Chem.* **73**, 483.  
Salmon, Guarrant and Hays (1928). *J. Biol. Chem.* **76**, 487.  
Sherman and Axtmayer (1927). *J. Biol. Chem.* **75**, 207.  
Smith and Hendrick (1926). *U.S. Treasury Pub. Health Rep.* **41**, 201.  
Stenbook, Sell and Nelson (1923). *J. Biol. Chem.* **55**, 399.  
Williams and Watorman (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 1.

## CII. STUDIES ON LIPIN-PROTEIN COMPLEXES.

### I. LECITHIN-CASEINOGEN COMPLEXES.

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*(Received January 28th, 1928.)*

It has long been known that lipins and proteins form loose "compounds" with each other, but up to the present no certainty seems to have been reached as to whether the constituents of these compounds are united merely by surface forces or whether their association is of a more intimate chemical nature. Galeotti and Giampalmo [1907] attempted to decide between these alternatives. They found on mixing lipins and proteins in various widely differing proportions that the composition of the resulting precipitates (as judged by the nitrogen content) although varying between only relatively narrow limits was not sufficiently nearly constant to enable them to deduce with certainty the formation of any definite chemical compound. In the same year, however, Mayer and Terroine [1907] studied the mutual interaction of lecithin and albumins and as a result stated definitely that the precipitates obtained on acidifying mixtures of these substances are colloidal complexes. Later Feinschmidt [1912] investigated the precipitation of mixtures of lecithin and various proportions of serum-proteins in solutions of graded acidity, and showed that the optimum hydrogen ion concentration at which the maximum precipitation of such mixtures occurs lies between the respective optimum values observed for the separate constituents alone. Meanwhile Hardy and Gardiner [1910] and Handovsky and Wagner [1911] pointed out the occurrence of lipin-protein complexes in blood-serum, or at least in the precipitates obtained therefrom by the addition of acids or salts, and following this Chick [1914] showed that the euglobulin precipitated by these reagents from serum is itself such a lipin-protein complex. Michaelis [1926] quotes a typical experiment of the kind described by Feinschmidt, and on repeating this we noticed that the precipitates produced in the relatively more acid solutions tended to float to the surface of the liquid, and so appeared to contain a larger proportion of the lighter lipin constituent than those produced in the less acid range, which were seen to settle more quickly to the bottom. As we had already been confronted with the question [Parsons and Parsons, 1927] as to the extent to which the lipins and proteins of serum are united into complexes, and as

to the conditions of precipitation of these complexes by salts, it seemed desirable to undertake a detailed investigation of the formation and properties of such complexes in general, and it is the object of the present paper to record the results of the first experiments made to this end.

In these experiments it was decided to use caseinogen as the protein constituent, on account of the ease with which it can be obtained pure (and, in particular, fat-free), while Merck's lecithin from eggs was chosen as the lipin component. In order to render possible the completion of the determinations on any one series of mixtures within a practicable length of time it was necessary to employ colorimetric methods of analysis. In order to obtain such a method for the estimation of the amount of lecithin present in a precipitated complex we stained this substance before use with a lipin-soluble but water-insoluble dye. When a precipitate was to be analysed it was first treated with a suitable volume of absolute alcohol in which the lecithin and the dye were at once dissolved. A measure of the amount of lecithin in this solution was then obtained by comparing the depth of its colour with that of an alcoholic solution of a known amount of the original stained lecithin preparation. Sudan III proved to be a suitable dye for this purpose. In order to obtain a uniformly stained lecithin preparation the lipin and a suitable small quantity of the dye were dissolved together in a little ether which was then allowed to evaporate. The resulting stained lecithin was then employed for the preparation of the emulsions used in the experiments described below. Of course it is admitted that the presence of the dye might to a certain extent influence the colloidal behaviour of the lecithin; but that such an influence is of appreciable magnitude in these experiments is hardly to be expected, on account of the smallness of the quantity of dye used, and this expectation is further justified by our observation that the point of maximum precipitation of the stained lecithin in the absence of protein corresponds with the hydrogen ion concentration at which the unstained lecithin itself is most readily precipitated from its emulsions. We intend to investigate further the question of the possible small effect of the dye by comparing the quantitative behaviour of deeply stained with lightly stained preparations and by the use of various staining materials. But meanwhile the experimental results recorded below must be understood to refer to lecithin containing the small quantity of Sudan III requisite for its estimation by the method outlined above.

For the colorimetric estimation of the caseinogen in the precipitates the method devised by Wu [1922] for the estimation of serum-proteins was found to be applicable. In this a phosphomolybdotungstate reagent is used which gives a blue colour with phenols generally, and so with the tyrosine of the protein molecule. The protein remaining after the complete alcohol extraction of the lipin from the original precipitate was dissolved in an appropriate volume of saturated sodium carbonate solution. After suitable dilution of this liquid the phenol reagent was then added, and when the resulting colour had

acquired a suitable and practically constant intensity it was compared with that obtained in a standard caseinogen solution treated in the same way simultaneously with the unknown. As a practical detail it may be mentioned that for a quantity of caseinogen of the order of 0.002 g. it was convenient to use 1 cc. of saturated sodium carbonate solution, to dilute then with 5 cc. of distilled water and to add 0.1 cc. of Wu's reagent.

In the first place it was decided to obtain information as to the variation of composition of the lecithin-caseinogen complexes precipitated from mixed colloidal solutions of these substances at hydrogen ion concentrations intermediate between their respective isoelectric points. The starting materials were a 0.4 % solution of caseinogen made up in 0.1 *N* sodium acetate according to the directions given by Michaelis [1926] and a 1 % emulsion in distilled water of Merck's lecithin stained with Sudan III. The necessary range of hydrogen ion concentration was obtained by the use of lactic acid-sodium lactate buffer solutions also made up according to Michaelis, and contained in 30 cc. portions in centrifuge tubes. To each of one series of tubes were added 3 cc. of a mixture of 1 volume of the lecithin emulsion with 2 volumes of the caseinogen solution. For comparison, each tube of a second similar series received 3 cc. of a mixture of 1 volume of lecithin emulsion with 2 volumes of 0.1 *N* sodium acetate solution while a third series was made up with 2 cc. of caseinogen solution + 1 cc. of distilled water in each tube. Thus the total volume of fluid and the concentration of sodium acetate were identical for all the tubes in the three series.

After standing for a period of 48 hours the precipitates of lecithin-caseinogen complex in tubes  $p_H$  3.6 to 1.4 were aggregated by light centrifuging and the supernatant liquids were cautiously sucked off. Each precipitate was then extracted with two successive portions of 5 cc. of absolute alcohol, the alcoholic extracts were carefully decanted from the caseinogen residues and the depths of colour of the combined extracts were compared in tubes of equal diameters with those of a series of standards prepared by suitable dilution of an alcoholic solution containing in 10 cc. the same amount of stained lecithin as had been added to each of the buffer solutions. The caseinogen residues were dissolved each in 2 cc. of saturated sodium carbonate solution + 10 cc. of distilled water, 0.2 cc. of Wu's phenol reagent was added to each tube and after an hour's standing the resulting blue colours were compared in a similar way with those produced in a series of suitable dilutions of the original caseinogen solution simultaneously treated in the same fashion. The results of these determinations, together with those of the similar ones carried out on the pure lecithin and caseinogen precipitates obtained in those tubes that received these substances alone, are represented graphically in Figs. 1 and 2, of which the first shows the actual amounts of lecithin and caseinogen precipitated together at the various hydrogen ion concentrations and the second the increase in the percentage of lecithin in the precipitate with increasing acidity of the solution. It will be seen that the change in composition of the

precipitated complex is really very considerable over the range investigated, the percentage of lecithin increasing from 27 at  $p_H$  4.2 to 100 at  $p_H$  1.4. The increase, however, is not regular, for over the intermediate portion of the range—from  $p_H$  3.6 to  $p_H$  2.9—the percentage of lecithin remains practically constant at a value of about 55. By comparing the two figures it will be seen that this range of constant composition of precipitate corresponds with the range over which all the lecithin and caseinogen present are thrown out of solution. It is conceivable, therefore, that over this range the caseinogen was capable of combining with more lecithin than was actually present, and that

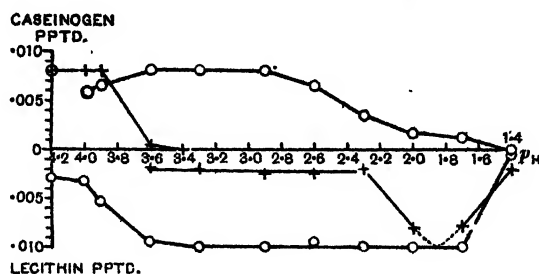


Fig. 1.

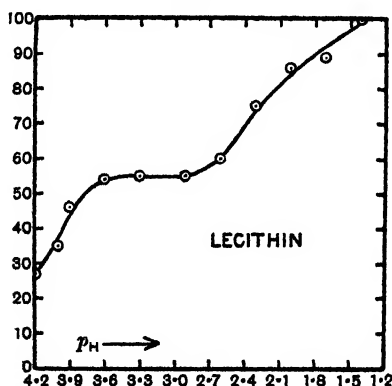


Fig. 2.

if we had started with a rather larger proportion of the lipin constituent this region of constant composition of precipitate, and with it the inflexion in the curve of Fig. 2, would have been absent.

It is probable that we are dealing here with a case of the mutual precipitation of two oppositely charged colloids, a phenomenon that has been familiar since the investigations of Biltz [1904]. In this case all the solutions which we used possessed hydrogen ion concentrations greater than that corresponding to the isoelectric point of the caseinogen so that this carried a positive charge, while the lecithin in the same solutions was on the alkaline side of its isoelectric point and so carried a negative charge. Presumably when the oppositely charged colloids are present together in solution the charge on the particles of one tends to be neutralised by the attraction of oppositely charged particles

of the other, and, when this process of neutralisation has resulted in the formation of complexes whose charge, although not necessarily zero, is too small to keep them in solution by mutual repulsion, precipitation occurs. In order to arrive at a quantitative explanation of the results of this experiment it is necessary to possess also some information as to the manner in which the charges on the two colloids which we have employed vary with the hydrogen ion concentration of the dispersion medium. In the case of the caseinogen it may be supposed that the charge on its particles is derived by a process of ionisation and that this ionisation is the more complete (or the activity of the caseinogen ions is the greater) the more remote the hydrogen ion concentration of the solution from the isoelectric point. But we should expect that once a certain hydrogen ion concentration has been reached the ionisation would be complete and that further increase of acidity would be without appreciable effect on the charge on the caseinogen particles. With regard to the lecithin there seems to be less evidence as to how the charge on its particles varies in relation to the reaction of the medium of dispersion. In this case the charge is possibly determined to a great extent by factors other than ionisation so that it might be expected to increase continuously with diminution of hydrogen ion concentration of the solution in a manner somewhat similar to that recently demonstrated by Michaelis and Damboviceanu [1924] in the case of mastic particles. If we assume that such relationships apply to the two colloids here under investigation it is possible to explain the proportions in which they mutually precipitate one another at various reactions. For in the less acid solutions the caseinogen particles will be but feebly positively charged while the lecithin will carry a relatively high negative charge. In this case, therefore, the adsorption of a comparatively small amount of the lipin will suffice to reduce the charge on the caseinogen particles to a point at which the complex will be precipitated. Thus the complex precipitated at these reactions will be rich in caseinogen and correspondingly poor in lecithin. On the other hand in the more acid solutions, the charge on the lecithin being less and that on the caseinogen being greater, relatively more lecithin will be required for neutralisation to the precipitating point and the complex obtained under these conditions will consequently contain a high proportion of lecithin. And this is indeed what is actually found to be the case. At first sight it would seem that on this theory the change of composition of the precipitated complex must be continuous over the range of hydrogen ion concentration between the isoelectric points of the two constituent colloids but since exact neutralisation of charge is probably not essential to mutual precipitation it is easy to account for the precipitates of practically constant composition obtained over a certain range of hydrogen ion concentration on the ground that not all of them were exactly isoelectric. In the more acid solutions the lecithin is too feebly charged to bring down more than a small quantity of the caseinogen; in fact in these solutions the lipin is precipitated when present alone and the protein appears

to exert no protective action on it. On the other hand in the least acid solutions the lecithin appears to be so strongly adsorbed by the caseinogen that a reversal of charge takes place and the resulting complex is therefore sufficiently negatively charged to remain in dispersion, for less of the protein is precipitated in the presence of the lipin than would be precipitated in its absence, a protective action being observed in this case.

We obtained further information regarding the mutual precipitation of these colloids by studying the composition of the complexes obtained when they were added in widely differing proportions to a buffer solution whose hydrogen ion concentration was so far intermediate between those corresponding to the isoelectric points of lecithin and caseinogen respectively that in it neither of these constituents was precipitated if present alone. For this purpose a lactic acid-sodium lactate mixture of  $p_H$  2.7 (standardised electrometrically) was chosen. As before we used a 1 % emulsion of stained lecithin in water and a 0.4 % solution of caseinogen in 0.1 *N* sodium acetate solution. 1 cc. of a mixture of 1 volume of the lecithin emulsion + 2 volumes of the caseinogen solution was first prepared: this gave a mixture denoted by the symbol  $\frac{L}{1}$  which has the same composition as that used for the experiment just described. A second mixture,  $\frac{L}{2}$ , was made by adding 1 cc. of a twofold dilution of the original lecithin emulsion to twice its volume of caseinogen solution, and by using further successive twofold dilutions of the emulsion other mixtures,  $\frac{L}{4}$ ,  $\frac{L}{8}$  and  $\frac{L}{16}$ , were prepared containing the same concentration of caseinogen throughout but diminishing quantities of lecithin equal respectively to  $\frac{1}{4}$ ,  $\frac{1}{8}$  and  $\frac{1}{16}$  of the amount present in mixture  $\frac{L}{1}$ . A second series of mixtures,  $\frac{C}{2}$ ,  $\frac{C}{4}$ ,  $\frac{C}{8}$ ,  $\frac{C}{16}$ , was also prepared using a constant amount of lecithin and twice its volume of successive twofold dilutions with 0.1 *N* sodium acetate solution of the original caseinogen dispersion, so that a total series was obtained in which the relative ratio of lecithin to caseinogen varied from  $\frac{1}{16}$  to 16. Of each mixture in the middle of the series 1 cc. was added to 10 cc. of buffer solution, but of the more extreme mixtures,  $\frac{L}{8}$ ,  $\frac{L}{16}$ ,  $\frac{C}{8}$  and  $\frac{C}{16}$ , 3 cc. were added to 30 cc. of buffer solution in order to obtain a larger amount of precipitate for analysis. It was noticed that the mixtures containing the greatest proportion of lecithin precipitated first. After a period of 48 hours the precipitates were aggregated by light centrifuging and analysed by the methods already described, care being taken to treat the standards identically with the unknowns and to obtain as accurate readings as possible by making the comparisons by means of a colorimeter.

From the results so obtained we calculated the amounts of caseinogen and lecithin precipitated in the various complexes per unit quantity (0.0033 g.) of total lecithin originally added to the mixture: these quantities are plotted in Fig. 3 in which positive ordinates represent g. of caseinogen, and negative



ordinates g. of lecithin, while the proportions in which these two constituents were added to the buffer solution are indicated by equally-spaced abscissae, *i.e.* by distances proportional to the logarithms of the actual numerical ratios concerned. The total amounts of the constituents either originally taken or appearing in the precipitated complexes are expressed by the correspondingly labelled curves.

Fig. 3 furnishes additional evidence in support of the view previously put forward that precipitation of the lecithin-caseinogen complex takes place only when the two components have combined in such proportions that mutual neutralisation of their opposite electric charges occurs to an extent sufficient to reduce the final charge on the particles of the total complex, if not neces-

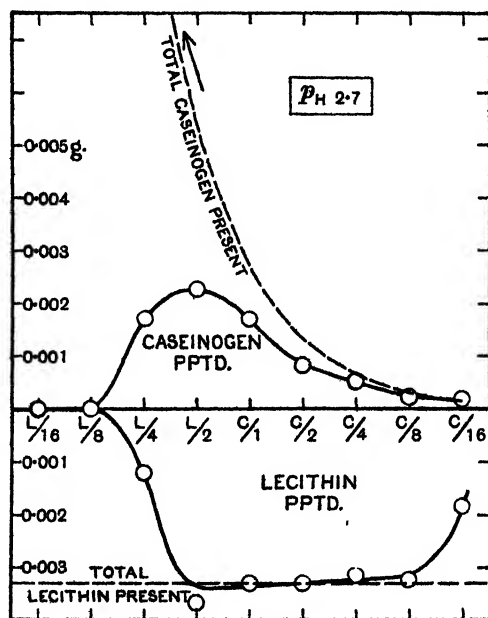


Fig. 3.

sarily to zero, at least to a point below a certain critical value. The first feature to be noted is that at the highest caseinogen concentrations (on the left of the figure) no precipitation at all of either component occurs. In this region we may suppose that the complex formed in solution contains so high a proportion of the positively charged caseinogen that the mutual electrostatic repulsion even of those complex particles that are relatively most feebly charged is powerful enough to maintain the condition of permanent dispersion. We are evidently dealing here with the same phenomenon encountered by Mayer and Terroine-[1907] when they found that their lecithin-albumin complexes were soluble in excess of the protein constituent. As the amount of caseinogen present is diminished we come to a point at which precipitation of complex just begins. Here we must suppose that while most

of the particles of the colloidal complex are still too rich in caseinogen, and therefore too highly positively charged to be precipitated, a certain small fraction of them, containing a relatively smaller proportion of the protein than the others, carry less than the critical intensity of charge and so are precipitated. As we proceed further we encounter the, at first sight surprising, circumstance that as the total amount of caseinogen present is still further reduced the amount of the protein in the precipitate steadily increases, which evidently means that the reduction of the percentage of caseinogen in the complex formed over this range is more than compensated by the extra amount of precipitation permitted by the concomitant reduction of charge. This increased precipitation of caseinogen continues until a certain maximum amount is reached in a mixture which is the first from which the whole of the lecithin present appears in the precipitate. With this particular ratio of the constituents we therefore obtain the maximum total amount of precipitate. Beyond this point further reduction in the total amount of caseinogen present leads to a reduction in the amount of this component precipitated, until, in the smallest caseinogen concentrations we at last arrive at a point at which there is not a sufficient proportion of the protein in all of the complex particles to reduce the negative charge due to the lecithin to below the negative critical precipitation value, so that only the particles relatively poor in lecithin are precipitated and a portion of this substance now again remains in solution. Finally we approach the condition in which practically no caseinogen is present, and no lecithin is precipitated.

Somewhere between the point at which all the added lecithin just begins to appear in the precipitate combined with the maximum amount of caseinogen, and that at which, in the mixtures poor in caseinogen, the whole of the lecithin just ceases to be precipitated, there must occur the isoelectric complex in which the charge, although not necessarily zero for all particles has a minimum average value. But from the results of this experiment alone it is not possible to discover which this ratio is. We are not justified in assuming that the isoelectric ratio is that in which the constituents are combined when the total weight of the resulting complex precipitate is a maximum, *i.e.* in the precipitate in which the whole of the lecithin appears associated with the maximum amount of caseinogen. For, as we show in Fig. 5, this precipitate does not contain a maximum *percentage* of caseinogen; it is but one of a series of precipitates whose caseinogen percentages diminish in a regular continuous fashion, so that all we can say is that the particles of this particular complex are, on the whole, less powerfully positively (or more powerfully negatively) charged than the particles of the complex obtained from mixtures richer in caseinogen, and conversely more strongly positively (or less strongly negatively) charged than those given by succeeding mixtures poorer in caseinogen.

An experiment was also carried out in which lecithin-caseinogen mixtures in the same ratios as those used in the experiment just described in detail were added to a less acid (lactic) buffer solution of  $p_H$  3.7 in which caseinogen

itself is precipitated when present alone. The results of this experiment in general confirm the deductions already drawn from a study of the behaviour of these constituents in the more acid solution. Attention is directed to the differences in detail produced by the circumstance that the caseinogen in the medium of  $p_H$  3.7 is less powerfully positively, and the lecithin more powerfully negatively, charged than in the solution previously used. In the first place it was noted that at  $p_H$  3.7 the mixtures containing the greatest proportion of caseinogen precipitated first. Further, the precipitate obtained from any given mixture in the less acid medium contained a lower percentage of lecithin, and therefore a higher percentage of caseinogen than the complex given by the same mixture in the more acid solution, as would indeed be expected from the results of the analyses of the precipitates obtained from the same lecithin-caseinogen mixture over a wide range of hydrogen ion concentration. In the mixtures richer in lecithin less of this substance was carried down in the complex than was the case with the corresponding mixtures in the more acid medium; on the other hand from the mixtures poorer in lecithin a relatively greater proportion was precipitated, but the effect of this on the final

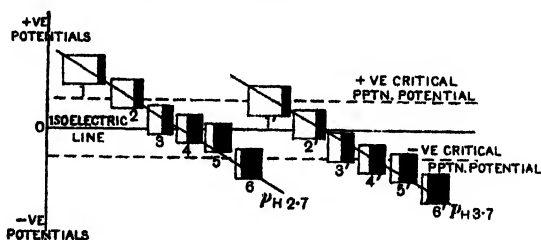


Fig. 4.

percentage of lecithin in the precipitate was more than counterbalanced by the much greater proportion of the added caseinogen that was also carried down. All these differences are readily explained when it is remembered that in the less acid solution a relatively much larger quantity of the now feebly charged caseinogen is required to reduce the high charge on the lecithin particles to the critical precipitation value.

Fig. 4 presents these relationships. Here the ordinates express the charge on the colloidal complex, positive or negative, as the case may be, the zero line indicating the completely discharged or isoelectric condition. The lines drawn parallel to and respectively above and below this represent the critical values of positive or negative charge which are just sufficient to keep the colloidal particles in permanent dispersion. In other words precipitation can occur only of particles carrying a charge whose magnitude is expressed by some point occurring between these two lines. The black portions of the rectangles are imagined as being proportional in area to the amounts of lecithin, and the white portions to the amounts of caseinogen, associated in the formation of complexes that may either be precipitated or remain in dispersion. It is not necessarily the case, nor indeed is it likely, that the

whole quantity of each substance added to a given mixture is used for the formation of such complexes; some of either constituent may remain free, but for the sake of simplicity of representation it is assumed that the whole of the lecithin and regularly progressing quantities of caseinogen are so combined. This assumption merely simplifies but does not invalidate our argument. The height of each rectangle is intended to express the (supposedly constant) range of variation of charge on the complex particles which it is regarded as containing. By following the figure from left to right it is easy to see how the complexes richest in caseinogen are devoid of particles whose charge is below the positive critical precipitation value (rectangle 1); how the first complex particles to be precipitated are still relatively rich in caseinogen (rectangle 2); and how it comes about that the greatest total amount of precipitate and of caseinogen carried down are associated with a complex whose caseinogen percentage is relatively smaller (rectangle 3). Finally by comparing rect-

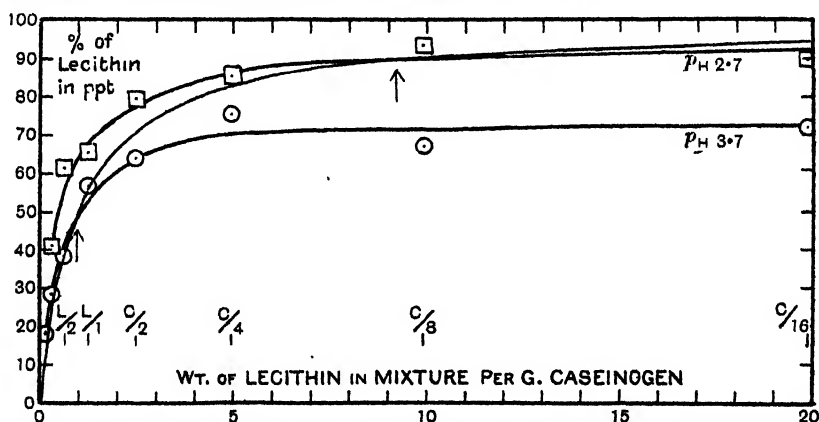


Fig. 5. The arrows indicate the compositions of the mixtures that are precipitated unchanged at  $p_H$  2.7 and  $p_H$  3.7 respectively.

angles 3, 4 and 5 it will be seen how complete precipitation of all the lecithin present may occur in complexes of different compositions, although only one of these (represented by rectangle 4) is really isoelectric, having the range of charge of its particles equally distributed about the zero line. Rectangle 6 represents the partial precipitation of a complex carrying a high negative charge on account of the presence of a large percentage of lecithin. The corresponding relationships for the less acid medium of  $p_H$  3.7 are represented by rectangles 1' to 6' in which a much larger percentage of the now feebly charged caseinogen is represented as being necessary for the reduction of the high charge on the lecithin to the critical precipitation value.

We pass now to a consideration of Fig. 5 in which the changes of composition of the precipitated complex observed in these experiments are plotted, in terms of percentages of lecithin as ordinates, against the corresponding ratios in which the constituent substances were mixed expressed numerically (not logarithmically) as abscissae. From the resulting curves it is seen that after

the first rapid increase in the percentage of lecithin in the precipitate, further increase in the total amount of lecithin present in the solution produces less and less increase in the proportion of this substance in the precipitated complex. The occurrence of this long range over which the composition of the precipitated complex varies but little in response to very wide variations in the ratio in which its constituents are mixed readily accounts for the tendency on the part of some authors to regard this complex as a definite chemical compound, the small variations in the composition of the actual precipitate being ascribed to admixture of small and variable quantities of its constituents. But our observations show that this range of almost constant composition is but part of the continuous and in other ranges more pronounced variation in the composition of the complex that would be expected on purely colloid-chemical grounds.

#### SUMMARY.

1. The percentage of lecithin in the precipitates obtained by the addition of a mixture of lecithin and caseinogen to buffer solutions of graded acidity increases with increasing hydrogen ion concentration of the medium.

2. When mixtures of caseinogen and progressively increasing quantities of lecithin are added to the same buffer solution the increase, at first rapid, in the percentage of lipin in the several precipitates becomes more and more gradual until a stage is reached at which very considerable further increase of the total proportion of lecithin added produces but slight increase in the percentage of this constituent in the precipitated complex.

3. These results are explained in terms of the mutual precipitation of two colloids whose opposite charges vary in intensity with the hydrogen ion concentration of the dispersion medium.

I wish to take this opportunity of expressing my indebtedness to Mrs K. Pinhey for valuable help with these experiments.

#### REFERENCES.

- Biltz (1904). *Ber. deutsch. chem. Ges.* **37**, 1095.  
Chick (1914). *Biochem. J.* **8**, 404.  
Feinschmidt (1912). *Biochem. Z.* **38**, 244.  
Galeotti and Giampalmo (1907). *Arch. Fisiol.* **5**, 503.  
Hardy and Gardiner (1910). *J. Physiol. ; Proc.* lxxviii  
Handovsky and Wagner (1911). *Biochem. Z.* **31**, 32.  
Mayer and Terroine (1907). *Compt. Rend. Soc. Biol.* **62**, 398.  
Michaelis (1926). *Praktikum der physikalischen Chemie*, 3rd ed. p. 76. (Springer, Berlin.)  
Michaelis and Damboviceanu (1924). *Kolloid. Z.* **34**, 322.  
Parsons and Parsons (1927). *Biochem. J.* **21**, 1194.  
Wu (1922). *J. Biol. Chem.* **51**, 33.

# CIII. STRUCTURE AND ENZYME REACTIONS. PARTS V AND VI.

## THE SYSTEMS GLUCOSE-ENZYME AND ESTER-CATALYST.

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*(Received May 7th, 1928.)*

IN the introduction to Part I of this series [Przylecki *et al.* 1927] it was shown that the introduction of an adsorbent into certain enzyme-substrate systems diminishes the velocity of reaction in those cases where both enzyme and substrate undergo adsorption. As was shown in Part III [Przylecki and Niedzwiecka, 1928] retardation of reaction may also be observed in systems where the presence of an adsorbent leads to a reduction in the number of collisions taking place in unit time between enzyme and substrate particles. Similarly, it might be conceived that insufficient stirring or agitation of the suspension of adsorbent on which either the enzyme or the substrate is adsorbed could reduce the velocity of reaction. As was shown in Part I, this phenomenon is not of general occurrence and depends upon the velocity of reaction and rate of diffusion for each particular case; where the latter factor is negligible in comparison to the former, no retardation should be observed.

The previous papers of this series deal with practically irreversible reactions, whose velocity is proportional to the concentrations of enzyme and substrate, whilst the products of reaction are adsorbed to a considerably less extent than the substrate. The latter factor, while of no great importance in the case of irreversible reactions, may become of importance in those cases where only the products of reaction are adsorbed. In such cases, the presence of an adsorbent may lead to an acceleration in the velocity of the later stages of an enzyme reaction, since the velocity constant may be expressed

$$K = \frac{[\text{enzyme-reaction products}]}{[\text{enzyme}] [\text{reaction products}]}$$

Thus a part of the products of reaction is separated by adsorption from chemical combination with the enzyme, leaving the latter free to combine with the substrate.

An adsorbent might therefore be chosen such that it not only does not retard, but even accelerates, irreversible reactions, and this acceleration will be greater the greater the value of  $K$ , i.e. the greater is the concentration of the products of degradation and the smaller that of the initial substance.

In Part I the possibility was raised of the acceleration of reversible reactions and of the shifting of the equilibrium point under the influence of an added adsorbent. This might occur in those cases where either the products of synthesis or of degradation are differentially adsorbed. The following conclusion, based on the law of mass action, might hence be drawn, only those cases being considered in which the adsorption of the substrates is reversible, and in which the adsorbent does not enter into chemical reaction with the substrates.

The kinetics of enzyme reactions would here depend upon two factors—the shifting of the point of equilibrium and the degree of adsorption. Where the degrees of adsorption of substrates and products are similar, both when the products of synthesis or of degradation are present in excess, the velocity of reaction diminishes, as a result of the removal by adsorption of the substrates from the sphere of action of the enzyme. If, however, the products of synthesis are adsorbed to a greater extent than the substrates the reaction will depend kinetically upon its relation at any given moment to the definitive equilibrium point, *i.e.* upon the quotient

$$K = \frac{[\text{products of degradation}]}{[\text{products of synthesis}]}$$

Where the products of synthesis are present in excess, so that the reaction is proceeding in the direction of degradation, the addition of an adsorbent should retard the reaction, as in the case of the system amylase-charcoal-polysaccharide (Part II). Should, however, the reaction of synthesis be dominant several possibilities might arise.

If the substrates of the reaction of synthesis do not undergo adsorption, whilst at the same time the adsorbed enzyme retains its activity, the reaction should be accelerated almost from the commencement, since the products of synthesis are being continually removed by adsorption from the reaction mixture. The difference between reaction in a homogeneous and a heterogeneous system will be greater the closer the former system is to its equilibrium point, and the quantity of synthesised product present at any one moment in the former system after the attainment of equilibrium will be smaller than in the latter. In such a case, the value of  $K$  for the system as a whole diminishes with increasing adsorption of the synthesised products, although in the disperse phase alone it will not have undergone any change, except that the concentrations of all the constituents will be smaller.

In those systems, however, in which both the substrates and synthesised bodies undergo adsorption, but the latter to a greater extent than the former, conditions are more involved. Whilst, in the above case, the velocity of the reaction of synthesis is almost from the beginning greater in the presence than in the absence of an adsorbent, in the present case two stages of the reaction may be distinguished. In the first stage, the concentration of substrates in solution is, as a result of their adsorption, smaller than in a homogeneous system, whilst the adsorption of products of synthesis is too small noticeably

to affect the velocity of reaction. As a result, the addition of an adsorbent to a system at this stage may retard the reaction of synthesis. The second stage supervenes when the reaction is approaching equilibrium; here adsorption of the substrates is insignificant, but that of the synthesised products is so large as to cause acceleration of the reaction. Graphically, we would here obtain two curves—one rising steeply but soon becoming flatter, and the second at first less steep, but retaining its initial slope longer, so that eventually it cuts the former curve and rises above it. This reasoning applies, of course, only to reactions for which the velocity of reaction is a function of the concentration of the substrates. In a system in which the latter are present in excess, or are very feebly adsorbed, the adsorption of a part of them need have no perceptible effect upon the velocity of reaction in its initial stages. The kinetics of such a reaction would be unaffected by the addition of an adsorbent.

In the case of certain biological syntheses, the application of the above illustrations may be exceedingly involved. Thus, for such heterogeneous reactions as the synthesis of glycogen where the eventual product of synthesis undergoes adsorption, and the products of the various stages of synthesis are located in different, spatially separated parts of the cell, the velocity of reaction would be diminished by the presence of an adsorbent. This, however, need not apply to a reaction of synthesis catalysed by a number of enzymes, in which the whole process takes place in one part of the cell, whilst the intermediate products are relatively not adsorbed to any great extent. Whether the synthesis of polysaccharides takes place under such conditions it is not yet possible to decide, as our knowledge of this process is insufficient. The biological synthesis of fats is, however, in all probability of this type. Proteins are possibly synthesised in a system of the first type, in which the reaction proceeds in several stages, spatially separated from each other. Little consideration has, however, up to the present been given to this problem.

A third general possibility is of the adsorption of the products of degradation being greater than that of the substrates. In such cases, in which the synthesised body does not undergo adsorption and is present in excess, the addition of an adsorbent should accelerate the reaction of degradation almost from the beginning, whilst, where the product of synthesis undergoes adsorption, parallel conditions obtain to those found in certain systems of the second type, *i.e.* the velocity of degradation would be retarded in its initial and accelerated in its final stages. In systems of this type, the velocity of synthesis should be diminished by the addition of an adsorbent, which would lower the concentration of substrates.

Thus the addition of an adsorbent to systems of the type enzyme-products of synthesis-products of degradation, in which the enzyme acts practically reversibly, may cause the following changes.

1. *In the equilibrium point.*  $K$  remains unaltered in the disperse phase, but in the system as a whole, including those of its constituents present in the



adsorbed condition, it is either changed in favour of the reaction of synthesis, should the products of the latter be more strongly adsorbed than the substrates, or in favour of the reaction of degradation, should the contrary be the case.

2. *In the velocity of reaction.* This may remain unaltered, be retarded or be accelerated.

The influence of the adsorbent on these two factors,  $K$  and velocity of reaction, has been expressed mathematically by Sym, as follows.

If  $a$  be the concentration of the reacting substance in the absence of an adsorbent, then after the addition of the latter the concentrations in solution and on the adsorbent will be together  $a_1 + \frac{a}{m} a_1^{1/n}$ , whilst at some time during the course of the reaction, they will be

$$a_1 - x + a/m (a_1 - x)^{1/n} + x_1 + \beta/m \cdot x'^{1/p},$$

and the quantity of substance  $y$  which has entered into reaction would be given by the equations

$$y = x' + \beta/m x'^{1/p} = x + a/m [a_1^{1/n} - (a_1 - x)^{1/n}].$$

The velocities of reaction and the equilibrium constants would then be as follows, for systems in which adsorption is reversible and mutually independent for each constituent.

1. In the absence of an adsorbent,

$$\frac{dy}{dt} = k_1 (a - y) - k_2 y, \text{ and } K_1 = \frac{a - y}{y}.$$

2. Adsorption of the reacting substance,

$$\frac{dy}{dt} = k_1 (a_1 - x) - k_2 y, \text{ and } K_1 = \frac{a - x}{y}.$$

$$K_2 = \frac{(a_1 - x_1) + a/m (a_1 - x_1)^{1/n}}{y_1}.$$

3. Adsorption of product of synthesis,

$$\frac{dy}{dt} = k_1 (a - y) - k_2 x', \text{ and } K_1 = \frac{a - y_1}{x_1},$$

$$K_3 = \frac{a - y_1}{x_1' + \beta/m \cdot x_1'^{1/p}}.$$

4. Adsorption of both substances,

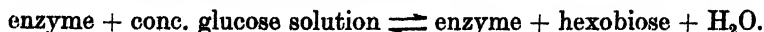
$$\frac{dy}{dt} = k_1 (a_1 - x) - k_2 x', \text{ and } K_1 = \frac{a_1 - x_1}{x_1'},$$

$$K_4 = \frac{a_1 - x_1 + a/m (a_1 - x_1)^{1/n}}{x_1' + \beta/m \cdot x_1'^{1/p}}.$$

In view of the great number of enzymic reactions which take place in the living organism, often reversible, and whose substrates, products or enzymes are adsorbed by substances found within the cell, the above theoretical reasoning calls for experimental verification. A number of researches with this end in view are now being conducted at this laboratory; in this paper the results obtained for two systems will be described. These are the systems

enzyme-glucose-charcoal and hydrochloric acid-aliphatic acid-alcohol and ester-charcoal.

In the first system we have the reaction:



The reaction attains a definite equilibrium point the value of which, in systems without charcoal, depends upon the initial concentration of glucose. This system can be used for the demonstration of the postulate that structure may influence reaction by changing the value of  $K$  and so augmenting synthesis, since the hexobiose formed is considerably more adsorbed than glucose, in solutions containing over 20 % of the latter and from 1 to 2 % of maltose.

The reaction, acid + alcohol  $\rightleftharpoons$  ester + water, proceeds under the catalytic influence of hydrochloric acid in a medium containing excess of alcohol. The addition to this system of preparations of charcoal, which adsorbs the organic acid to a considerable extent, but not the ester, brings about the acceleration of the reaction of hydrolysis.

#### THE SYSTEMS CHARCOAL-ENZYME-GLUCOSE AND ENZYME-GLUCOSE.

The work of Hill [1898], Emmerling [1901], Fischer and Armstrong [1902] and Bourquelot *et al.* [1913] has established that if an appropriate enzyme be added to a concentrated glucose solution, a reaction of synthesis of disaccharide proceeds to a certain equilibrium point.

This system has been selected as suitable for the experimental establishment of the hypothesis that the equilibrium point of reversible reactions may be shifted in favour of synthesis by the addition of a substance on which the products of synthesis are adsorbed to a greater extent than are the substrates.

A fundamental condition for this is the almost quantitative adsorption of the enzyme. Whilst from theoretical considerations it follows that the ratio of glucose to maltose in solution would be changed in favour of the former, a consideration of adsorbed glucose and maltose should indicate augmented synthesis of the latter.

A number of preliminary experiments had first to be undertaken:

(1) with a view to establishing that glucose and maltose are adsorbed by charcoal in concentrations approaching those which are obtained in the enzymic synthesis of maltose;

(2) for the analysis of these two sugars in adsorption on charcoal;

(3) in order to carry out a reaction lasting several months under sterile conditions, without the use of antiseptics, which would affect the relative adsorption of the sugars, and diminish their absolute adsorption;

(4) in order to elaborate a method for the quantitative determination of maltose in the presence of a large excess of glucose.

The researches of Bourquelot, of Emmerling and of Hill were of great assistance in the execution of our research. From the papers of these authors were taken the concentration of glucose used, the time of reaction, and the selection of the enzyme, as well as the method for the estimation of small quantities of disaccharide in the presence of a large excess of glucose.

A. *The equilibrium of the system enzyme-glucose.*

Our first experiments had as their objects the establishment of the necessary technique for working over long periods of time without the use of disinfectants; further, the achievement of conditions relatively close to those of final equilibrium in the absence of charcoal, and the determination of the degrees of adsorption and of elution, together with the conditions under which the latter takes place.

(a) *Sterilisation.* The elutive effect of ordinary antiseptics militated against their use in these experiments, and the following technique was therefore used for the maintenance of aseptic conditions. The glucose solutions, with or without charcoal, were sterilised by heating in an autoclave for 30 minutes in 250 cc. Erlenmeyer flasks stoppered with cotton-wool. The enzyme solutions were sterilised either by passage through a Chamberland filter or by the addition of powerful antiseptics such as ethyl alcohol, which were then removed under diminished pressure at 20°. The sterilised enzyme solutions were in some cases measured out from sterilised graduated cylinders and then added to the sterilised glucose solutions. In others, the flask containing the glucose solution was tightly stoppered with cotton-wool, through which passed two tubes, one connected to a filter pump and the other to a graduated vessel containing sterilised enzyme solution. The diminution in volume of the contents of the latter vessel represented the volume of enzyme solution added to the glucose solution. In many cases, as an additional precaution against infection, methyl alcohol was added. This is a fairly strong antiseptic, whilst at the same time its elutive power is inconsiderable.

The above measures enabled us to maintain sterile conditions for over 6-7 months. Before each determination, the solutions were tested for sterility, and only those from which micro-organisms were absent were taken for analysis.

(b) *Choice of enzyme.* Emmerling [1901] showed that isomaltose and dextrin are produced in concentrated solutions of glucose in the presence of yeast maltase, whilst Bourquelot *et al.* [1913] used several enzymes such as emulsin for the synthesis of disaccharides. In this research, yeast maltase prepared according to Emmerling was used.

(c) *Determination of synthesised disaccharide.* The method described by Emmerling was applied, with slight modifications. Glucose was completely decomposed by fermentation, and the remaining disaccharide hydrolysed by hydrochloric acid, the glucose thus formed being then determined by Bertrand's method. The fermentation was carried out by the agency of *Saccharomyces marxianus*, *S. exiguus* or *Willia anomala*.

(d) *Establishment of equilibrium.* Two points had here to be proved; firstly that the velocity of synthesis is almost unaffected by the addition of charcoal at the initial stages of the reaction, and secondly that the high degree of adsorption of maltose on charcoal shifts the equilibrium point of the system as a whole in the direction of synthesis. For the establishment of these facts absolute equilibrium was not essential, conditions close to this state being sufficient. Since solutions from which samples had been taken for analysis could not be used further, and as the entire contents of a flask had to be used for the determination of the degree of elution, the attainment of conditions close to equilibrium had to be judged by the determination in different flasks of the ratio of glucose to maltose at fortnightly intervals beginning a month after the commencement of the experiment. In this way, it was found that in the absence of charcoal initially 20 % glucose solutions contained at equilibrium 1.48 % of disaccharide (see Table I).

Table I. *Synthesis of hexobiose.*

Initial concentration of glucose (%)	Hexobiose found after 5 months (%)
20	1.29, 1.58, 1.71, 1.34
	Mean 1.48

B. *Adsorption of glucose and disaccharide on charcoal.*

Having determined equilibrium conditions in the absence of charcoal the system enzyme-20 % glucose could now be studied in its presence.

In order to obtain measurable differences in the equilibrium point of the reaction it was necessary to create conditions such that the quantity of adsorbed disaccharide was as great as practicable. This was done by introducing large quantities of adsorbent, the components of the systems examined being present in the following quantities: 5 g. of charcoal, 50–100 cc. of a solution containing 10–20 % of glucose and 1–2 % of maltose. The charcoal preparations used were either of local manufacture or Merck's "Carbo medicinalis." The mixtures were placed in a mechanical shaker for 4–6 hours, after which they were left in a thermostat at 37° for 12 hours, and then filtered. The results obtained are given in Tables II, III and IV. The values found show that barely 7.2 % of glucose is adsorbed on local charcoal from a 16.6 % solution, and 8.1 % from a 9.0 % solution. Maltose is adsorbed far more intensely, to the extent of 63.5 % from a 0.86 % solution, and 43.2 % from a 1.4 % solution. Considerably higher degrees of adsorption were observed for Carbo medicinalis, being 12 % from a 20 % solution of glucose and 97 % from a 2 % solution of maltose.

From the point of view of our experiments, it was of great importance to establish the degrees of adsorption of glucose and maltose in systems containing originally only the former sugar, to which maltose solution is added later. Such conditions would correspond to those obtaining during the enzymic synthesis of hexobiose in the presence of an adsorbent.

Table II. *Adsorption and elution of maltose and glucose.**Percentage concentrations.*

	Without charcoal	With charcoal			
		Before elution		After elution	
		In solution	% adsorption	In solution	% adsorption
Glucose solution	9.0	8.29	8.1	9.0	0
Maltose solution	0.86	0.31	63.5	0.70	18.6

Table III. *Adsorption of glucose and maltose separately and together.**Percentage concentrations.*

Solution				Without charcoal	With charcoal	% adsorption
Glucose	...	...	...	16.6	15.4	7.2
Maltose	...	...	...	1.4	—	43.2
Maltose 1.4 %, with glucose 16.6 %	...	...	...	—	—	33.7

90 cc. of 22 % glucose were added to 5 g. of charcoal, the whole was shaken 3 hours and left overnight. 10 cc. of 2 % maltose solution were then added drop by drop and with constant shaking, an interval of one hour being allowed after the addition of each 2 cc. The results obtained are given in Tables III and IV.

Table IV. *Adsorption and elution of glucose and maltose together and separately.**Percentage concentrations.*

Solution				Without charcoal	With charcoal			
					Before elution		After elution	
					% in solution	% adsorption	% in solution	% adsorption
Glucose	...	...	...	20.11	17.7	12.0	19.3	4.0
Maltose	...	...	...	1.97	0.058	97.0	0.97	50.0
Maltose and glucose together	...	...	...	—	0.674	65.8	1.50	24

These values are based upon analyses and calculations of the concentrations of solutions of maltose with glucose with and without charcoal. Maltose was determined after the fermentative destruction of glucose by hydrolysis in the presence of hydrochloric acid, the glucose thus formed being then determined by Bertrand's method. The amount of adsorption of glucose was determined polarimetrically and was calculated from the difference between the optical rotations with and without adsorbent of the solution of maltose and glucose less that of the maltose alone. Comparative values are given only for the adsorption of maltose, which alone concerns us in the study of equilibrium conditions.

The adsorption of maltose in the presence of 16.6 % glucose on charcoal preparation No. 1 (Table III) is considerably smaller than in its absence. Thus adsorption is here diminished by 22.2 %, from 43.2 to 33.7 %. The

second charcoal preparation which normally adsorbs 97 % of maltose from solution exhibits an adsorption of maltose of only 66 % in the presence of 20 % glucose, a diminution of 31 %.

A number of experiments, the detailed results of which will not here be given, showed that the diminution of adsorption of maltose caused by the addition of glucose depends upon the concentrations of these sugars. Thus the greater the concentration of maltose used the more closely do the adsorptions of glucose and maltose separately approach each other, and the same is the case when the concentration of glucose is lowered. On the other hand, the difference in the degree of adsorption of maltose in the presence of glucose is intensified as the concentration of the former sugar rises, and this difference becomes even more accentuated in more concentrated solutions of glucose. These considerations possess a certain biological significance. Thus, changes in the enzymic equilibrium of this reaction will, up to a certain point, be the more marked the greater the relative concentration of glucose, and the augmented yield of biose due to the presence of an adsorbent will vary inversely as the concentration of maltose in equilibrium in its absence. These conclusions follow from the very similar degrees of adsorption of these two sugars. The attainment of the differential adsorption of glucose and maltose thus depends upon their concentrations being chosen so as to fall in different regions of the adsorption isotherms.

The above would be of considerably less importance in those cases in which the degree of adsorption of the synthesised body differs greatly from that of its progenitors, as in the case of glycogen and glucose, where glucose undergoes only negligible adsorption upon protein whilst that of glycogen is very considerable. In this case, the presence of an adsorbent may accelerate synthesis and change the equilibrium constant even with large concentrations of glycogen and small concentrations of glucose.

### C. *Elution of the sugars.*

Changes in the equilibrium point of this enzymic reaction are apparent only if the adsorbed biose be taken into consideration. Thus, for the determination of  $K$  it was necessary to find the sum of glucose and maltose adsorbed and in solution. This was done by elution of adsorbed sugars by means of amyl alcohol, which had been shown by Przylecki and Niedzwiecka [1928] to act as a powerful elutive agent of uric acid from charcoal. The procedure was as follows. Solutions of glucose and maltose separately or together were added to charcoal in the same order and applying the same precautions as described previously. Amyl alcohol was then added to the mixture, which was then mechanically shaken for 3-6 hours, left tightly stoppered for 12 hours, and again shaken for 3-6 hours. The mixture was then filtered into separating funnels, and the aqueous layer of the filtrate taken for analysis. The optical rotation of the solution of sugars, either mixed or separate, was now measured,

and the same analytical procedure applied as before to the determination of the concentrations of the individual sugars.

The determination of maltose in the presence of excess glucose and of amyl alcohol was with certain modifications effected according to Emmerling. In order to establish that the presence of amyl alcohol did not in any way influence the reaction of fermentation, the following experiments were carried out. Yeast maltase was added to the above filtrates, containing glucose and maltose separately or together. The mixtures were then heated, to drive off amyl alcohol and to destroy the hexobiose-synthesising enzyme, and the excess glucose was destroyed by fermentation.

The results obtained showed that no reducing bodies remained after the fermentation of glucose, and that when maltose was present the content of this substance did not diminish under these conditions. Emmerling's method is therefore applicable to this analysis.

The results given in Tables II and IV show that for the sugars taken separately glucose undergoes almost quantitative elution, whilst maltose is only partially eluted, 18.6 to 50 % of the total quantity remaining in adsorption. Where a mixture of maltose and glucose is taken, a far smaller quantity of the former remains adsorbed after the addition of amyl alcohol, the percentage adsorption falling from 65 to 24 %.

*D. Comparison of enzymic equilibrium in the presence and in the absence of charcoal.*

The technique employed in these experiments was similar to that used in the preliminary investigations. 50–100 cc. of 20 % glucose solution containing yeast maltase was added to 5 g. of charcoal, and analyses were made of the sugars after 1–2 weeks and 2–6 months. The relative concentrations of glucose and hexobiose were determined separately for the solution and for the whole system after elution with amyl alcohol. Glucose was determined either polarimetrically or by Bertrand's method as the difference between the total amount of glucose found after acid hydrolysis of the whole mixture and that produced by hydrolysis of the biose alone, which, as before, was isolated by fermentation of glucose.

Table V. *Synthesis of hexobiose in the presence of 5 g. of charcoal to 50–100 cc. of 20 % glucose.*

No. of determinations	Time	Volume of solution cc.	Without charcoal	With charcoal		Hexobiose after elution
				In solution	Reversibly adsorbed	
5	3–7 days	50	0.250	—	—	0.270
4	"	100	0.304	—	—	0.310
7	2 months	50	1.40	1.24	0.688	2.03
8	5 "	—	1.67	1.61	0.81	2.42

The results given in Table V afford a satisfactory experimental confirmation of the postulates put forward in the theoretical part of this paper. During

the first stages of the reaction, lasting 3-7 days, the presence of charcoal has no appreciable effect upon the velocity of the reaction of synthesis. Neither in the solution alone, nor in the whole system can any great difference be seen in the yield of synthesised hexobiose. If anything, it would appear that the amount of hexobiose synthesised in the presence of charcoal is smaller than in its absence, although this difference is so small as hardly to exceed the limits of experimental error.

This phenomenon appears to be due to the fact that at this stage the reaction is still far from its end-point, and its velocity should not therefore be greatly affected by the adsorption of the small quantities of hexobiose present. Similarly the removal by adsorption of glucose from solution, leading to a diminution in its concentration of from 18.6 to 20 % is unable greatly to diminish the velocity of the reaction of synthesis. As at this stage the very small quantities of hexobiose produced render its determination difficult, only its total amount in solution and adsorption was determined.

The results obtained after a lapse of 2-5 months are entirely different. Here very considerable differences exist between the amounts of hexobiose synthesised in the presence or absence of charcoal. Whilst in the latter case 1.40 % of biose is found after 2 months and 1.67 % after 5 months, in the presence of charcoal we find 2.03 and 2.42 % after 2 and 5 months respectively. This represents in the absence of an adsorbent a yield of 6.0 and 8.35 % respectively on the glucose used, whilst in the presence of charcoal the yields are 10.1 and 12.1 % respectively. The actual difference is undoubtedly even greater, since the uneluted hexobiose has not here been taken into consideration.

No attempt has been made to express the above differences in terms of  $K$ , since we have no certainty that definitive equilibrium has been attained—indeed, in those systems examined after 2 months there can be no doubt that equilibrium had not been reached, and for this reason the differences are expressed only as percentages. Thus, after 2 months the presence of charcoal increased the yield of hexobiose by 45 % and after 5 months by 44.1 %.

As to the solutions alone, the concentrations of biose are substantially the same whether an adsorbent is present or not. The cause of augmented synthesis lies solely in the charcoal, on which 0.688 % of the biose was adsorbed after 2 months, and 0.81 % after 5 months, corresponding respectively to 34.4 and 33.8 % of the total quantity synthesised.

#### INFLUENCE OF ADSORBENT UPON REACTIONS OF ESTERIFICATION.

The effect produced by the addition of adsorbent charcoal (Merck's) to systems containing alcohols, aliphatic acids and their esters, and hydrochloric acid as catalyst, was investigated.

The acid content was determined by titration with barium hydroxide solution, using phenolphthalein as indicator, and esters were determined by



hydrolysis with excess sodium hydroxide solution and back-titration with standard acid.

The procedure generally applied was as follows. Two flasks containing an aqueous solution of alcohol (solution III) of known concentration were taken, and a known quantity of aliphatic acid or ester was added to one flask (solution I). To the other a known quantity of hydrochloric acid was added (solution II); the concentration of alcohol in both flasks was kept equal.

After these solutions had been warmed to the reaction temperature of 37° the concentrations of their constituents was determined. Equal volumes of solutions I and II were then mixed, and the resulting solution IV analysed. The concentration of the constituents of solution IV amounted in every case to half the sum of those of solutions I and II separately. The time at which solutions I and II were mixed was noted, and represents the time of commencement of reaction.

To a portion of solution IV adsorbent charcoal was added immediately after mixing, and, after violent shaking over a short period, the degree of adsorption was determined in this mixture, VII. The degree of adsorption of the individual constituents of the latter mixture was determined by adding adsorbent charcoal to solutions prepared by mixing equal volumes of solutions I and III (solution V) or II and III (solution VI) and analysing after shaking. The sum of the quantity of compounds adsorbed from solutions V and VI was always close to that adsorbed from solution VII.

The concentrations of the substrates of solutions IV and VII were determined at various intervals of time up to the attainment of equilibrium, after which the degree of adsorption of the substrates and reaction products was determined.

10 cc. of solution was in every case taken for titration.

## RESULTS.

### I. *The system glycerol-butyric acid-charcoal-catalyst.*

Applying the same numeration as above, the following solutions were prepared:

- |      |                                     |                 |                                 |
|------|-------------------------------------|-----------------|---------------------------------|
| I.   | 400 cc. of glycerol (sp. gr. 1.233) | + 350 cc. water | + 50 cc. butyric acid.          |
| II.  | 400 cc.                             | „ „             | + 388 cc. „ + 12 cc. conc. HCl. |
| III. | 400 cc.                             | „ „             | + 400 cc. „                     |

The concentration of acid in solution I was equivalent to 336.9 cc. and in II to 88.6 cc. of 0.05 *N* barium hydroxide, half of the sum of which amounts to 212.7 cc., whereas that of solution IV was 212.3 cc. The concentrations of solutions V and VI were respectively 168.2 and 44.4 cc., after adsorption 137.5 and 44.4 cc. respectively, a total of 181.9 cc.

The concentration of hydrochloric acid in VI is thus unaffected by the addition of 2 g. of adsorbent per 100 cc. of solution. It was noticed incidentally that the filtrate from VI after the addition of charcoal gave a preci-

pitate of barium sulphate on the addition of barium hydroxide. Solution VII contained 182.2 cc. of 0.05 *N* acid, or only 0.3 cc. more than solutions V and VI together.

Table VI. cc. of 0.05 *N* butyric acid or ester.

Date ...	6. xii	7. xii	15. xii		16. xii		18. xii		$K = \frac{[\text{acid}]}{[\text{ester}]}$
	Acid	Acid	Acid	Ester	Acid	Ester	Acid	Ester	
without adsorbent	167.9	133.3	107.3	60.6	106.5	61.4	106.2	61.7	1.72
with adsorbent in solution	137.8	112.0	90.2	53.6	89.6	54.1	89.6	54.1	1.66
with adsorbent in adsorption	30.1	26.1	21.0	2.8	21.3	2.9	21.3	2.9	—
Total content	167.9	138.1	111.2	56.4	110.9	57.0	110.9	57.0	1.97

The results are given in Table VI. The quantity of adsorbed butyric acid was determined by direct titration of the residue left after filtering 200 cc. of solution VII, and deducting from the value obtained the quantity of acid contained in the solution absorbed by the filter-paper and between the grains of charcoal. Filtration of solution VII before the addition of charcoal showed that the filter-paper retains 5.6 cc. of 0.05 *N* acid, and it may be taken that the second item amounts to about 4 cc. of solution or about 36.0 cc. of 0.05 *N* acid. Since the filter-paper with residue after the filtration of 200 cc. of solution contains the equivalent of 469.0 cc. of 0.05 *N* acid, and the correction is altogether 41.6 cc., the figure of  $427.4/20 = 21.3$  cc. 0.05 *N* butyric acid is arrived at for the adsorption of this acid upon charcoal. The amount of adsorption of the ester is arrived at by subtracting from the difference between the acid + ester content with and without charcoal, namely

$$167.9 - 143.7 \text{ cc.} = 24.2 \text{ cc. } 0.05 \text{ } N \text{ acid + ester,}$$

representing adsorption of butyric acid and of its glyceride, the above value of 21.3 cc. found for acid alone, leaving a value of 2.9 cc. of 0.05 *N* ester adsorbed. Equilibrium in the heterogeneous system is thus represented by  $K = 1.97$ , as against a mean value of 1.69 for the dispersing medium alone. The introduction of an adsorbent into this system increases, therefore, the equilibrium constant, which is changed in favour of hydrolysis.

## II. The hydrolysis of ethyl acetate.

The procedure adopted for this system was identical with that of the preceding, except that the substrates were ethyl acetate dissolved in 44.4 % alcohol. The quantity of charcoal used here was 3 g. per 100 cc. of solution, and as before, 10 cc. of solution were in all cases taken for titration, using 0.0397 *N* barium hydroxide. Using the same numeration of solutions as previously, solutions IV and VII contained initially 93.5 and 82.5 cc. of 0.0397 *N* ester, and 7.9 and 7.5 cc. of 0.0397 *N* hydrochloric acid respectively. Equilibrium was attained in solution IV in 13 days, the initial and final conditions being illustrated in Table VII.

Table VII. cc. of 0.0397 *N* acetic acid or ester.

	Initial content of		Final content of		$K = \frac{[\text{acid}]}{[\text{ester}]}$
	Acid	Ester	Acid	Ester	
Without adsorbent	0	93.5	47.5	46.0	1.03
With adsorbent { in solution	0	82.5	43.2	41.3	1.02
{ in adsorption	0	11.0	1.7	7.5	—
Total content	0	93.5	44.9	48.8	0.92

The degree of adsorption of the compounds present in the equilibrium mixture is derived from the values found for each constituent separately at concentrations close to those found at equilibrium. Thus 7.4 cc. of 0.0397 *N* ethyl acetate were adsorbed from a solution containing 40.9 cc. Assuming that a certain proportionality exists between the amounts in adsorption and in solution for small changes in concentration, it is here taken that 7.5 cc. are adsorbed from a solution containing 41.3 cc. of 0.0397 *N* ester. Similarly for acetic acid 2.3 cc. were adsorbed from a solution containing 57.9 cc., whence 1.7 cc. would be adsorbed from the equilibrium mixture containing 43.7 cc. of 0.0397 *N* acetic acid. These calculated values are in satisfactory agreement with experimental values, as is shown by a comparison between the sum of adsorbed and unadsorbed acid and ester found in solution VII, and the value for the amount of ester present at the beginning of the reaction, namely the equivalent of 93.7 and 93.5 cc. of 0.0397 *N* barium hydroxide respectively.

In this case the degrees of adsorption of the acid and ester are different from those existing for the first reaction studied, and the equilibrium constant is therefore lowered by the addition of an adsorbent, *i.e.* the reaction of hydrolysis does not proceed as far in the presence as in the absence of an adsorbent. At the same time it will be seen that the equilibrium constant remains unaffected in the dispersing medium.

### III. The synthesis of ethyl acetate.

This system consisted of 12.4 % ethyl alcohol containing acetic acid, with hydrochloric acid as catalyst; 5 g. of charcoal per 100 cc. of solution were added. The results, expressed in terms of cc. of 0.0397 *N* barium hydroxide are given in Table VIII.

Table VIII. cc. of 0.0397 *N* ethyl acetate or acetic acid.

Date ...	2. iv HCl	2. iv Acid	3. iv Acid	4. iv Acid	5. iv Acid	6. iv Acid	16. iv Acid	18. iv		$K = \frac{[\text{acid}]}{[\text{ester}]}$
								Acid	Ester	
Without adsorbent	6.7	56.0	49.4	48.4	48.2	48.0	47.8	47.8	8.2	5.8
With { in solution	5.0	46.5	43.6	41.3	38.8	36.2	34.7	34.0	6.1	5.6
adsorbent { in adsorption	—	9.5	9.0	8.4	7.9	7.6	7.4	7.4	9.2	—
Total content		56.0	52.6	49.7	46.7	43.8	42.1	41.4	15.3	2.7

In this case a very considerable enhancement of synthesis has taken place as a result of the addition of charcoal. This is due entirely to adsorption, since the equilibrium attained in the dispersing phase ( $K = 5.6$ ) is little

different from that of the homogeneous system, for which  $K$  is 5.8. In the system as a whole, however, including that part of its constituents which is in adsorption, 7.1 cc. more of ester have been synthesised, representing an increase in yield of over 86 %, with a consequent diminution of  $K$  to 2.7. Curves illustrating this system are given in Fig. 1. It will be seen that synthesis, although at first more rapid in the absence of an adsorbent, soon slows down, and that the first portion of Curve II, representing reaction in the presence of charcoal, is practically a straight line. This would be due to

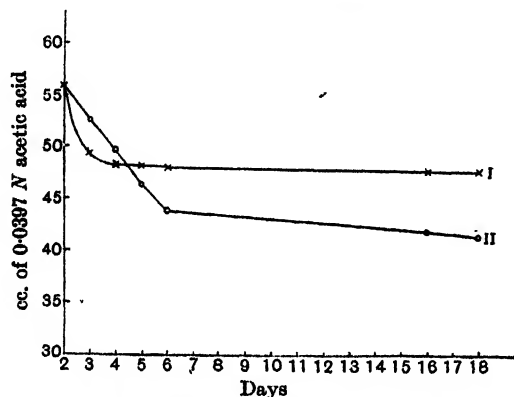


Fig. 1. Curves expressing concentration of acid in the absence (I) and presence (II) of adsorbent.

the practical absence of ester in the first stages of the reaction, which would only commence to exhibit retardation when the charcoal surface is under the given conditions close to saturation with ethyl acetate. The smaller initial velocity of reaction in the presence of charcoal is probably due to adsorption of acetic acid, as a result of which its active concentration is diminished.

The above three cases fully confirm the theoretical reasoning put forward in the first part of this paper and afford excellent examples of the mechanism of reversible reactions in the presence of adsorbents.

#### REFERENCES.

- Bourquelot, Herissey and Coirre (1913). *J. Pharm. Chim.* **8**, 441.  
 Emmerling (1901). *Ber. deutsch. chem. Ges.* **34**, 600.  
 Fischer and Armstrong (1902). *Ber. deutsch. chem. Ges.* **35**, 3144.  
 Hill (1898). *J. Chem. Soc.* **73**, 634.  
 Przylecki and Niedzwiecka (1928). *Biochem. J.* **22**, 34.  
 Przylecki, Niedzwiecka and Majewski (1927). *Biochem. J.* **21**, 1025.

## CIV. THE AMINO-ACIDS OF FLESH.

### II. COMPARISON OF THE DIAMINO-ACID CONTENT OF SOME NORMAL AND PATHOLOGICAL TISSUES.

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*(Received April 20th, 1928.)*

IN a previous paper [Rosedale, 1922] the diamino-acid contents of the muscle of various farm animals were compared. Very slight differences could be detected between the tissues analysed, although it was pointed out that available methods of analysis did not ensure complete accord even when duplicates were made upon the same hydrolysate. During the intervening years, fresh endeavours have been made to secure greater accuracy in the analysis of proteins. Plimmer [1924] studied more closely the quantitative reaction of the hexone bases with nitrous acid. Plimmer and Phillips [1924] estimated histidine and tyrosine, after hydrolysis, by bromination. Plimmer and Rosedale [1925] have investigated each step in the Van Slyke estimation method and published certain modifications which secure greater ease and accuracy in the procedure. More recently, Plimmer and Lowndes [1927] have devised a method by which cystine may be estimated in the diamino-fraction of the Van Slyke method. The work now presented was undertaken as an extension of the data previously obtained. With two exceptions, muscle had not been analysed from different parts of the same animal. It has also been thought desirable to compare the results of the Van Slyke method with the older procedure of Kossel. The latter method which includes so many manipulations cannot necessarily claim greater accuracy than other methods of estimation, although in the hands of Kossel and Osborne the results have proved sufficiently concordant to form the basis of our knowledge of the subject to-day. Certain modifications of Kossel's original procedure have been introduced which avoid some of the difficulties. The opportunity has been taken of comparing certain pathological with normal tissues, and of analysing the muscle tissue of animals which had been fed upon known proteins.

#### EXPERIMENTAL.

After removing as much fat as possible the tissues were minced. They were then extracted in 2 l. of boiling water containing 2 cc. glacial acetic acid in order to coagulate the protein and to separate it from extractives. The liquid was poured off and the tissue squeezed dry in a cloth. The procedure of extracting and drying was repeated twice. The tissues were then

digested for 8–12 days at 37° with 1 g. of pepsin in 2 l. of 0.1 N HCl. Indigestible matter was filtered off and the total nitrogen estimated by Kjeldahl's method. The filtrate was then hydrolysed with 25 % HCl for 36 hours, when it was evaporated to dryness *in vacuo* once or twice to remove as much HCl as possible. The hydrolysate was finally brought to such volume that 100 cc. represented approximately 0.5 g. of nitrogen. Portions of 100 cc. were then analysed by Van Slyke's method, according to the technique of Plimmer and Rosedale [1925], except in the cases of pig uterus and placenta when the original method was followed, although in these analyses also, the determinations of amide- and humin-nitrogen were carried out in accordance with the procedure of Plimmer and Rosedale, and the estimation of arginine-nitrogen followed the modification of Plimmer [1916] in every case.

The main difficulty in the Kossel procedure is that loss occurs at each precipitation. The problem has been therefore to ascertain whether any of the precipitates, especially those involving the formation of barium sulphate can be dispensed with. Various alterations have been attempted during the course of the work and the procedure finally adopted is as follows.

After removal of the portions for analysis according to Van Slyke, the bulk is acidified to 5 % with HCl and precipitated with phosphotungstic acid. The amount of phosphotungstic acid used was 15 g. for each 0.5 g. of nitrogen, the volume of the bulk being doubled in accordance with the Van Slyke procedure. The precipitation is carried out in a large flask which is heated in a water-bath to redissolve the precipitate. After standing for at least 48 hours, the precipitate, which contains, in addition to the diamino-acids, ammonia and humin, is filtered on a Büchner funnel and washed five times with 50 cc. of dilute HCl. The precipitate is then placed in a basin for the decomposition of the phosphotungstates, which is carried out by means of baryta gradually added with stirring until the solution is faintly alkaline. The precipitate of barium phosphotungstate, to which the humin adheres, is rapidly filtered off by suction and the filtrate which now contains the diamino-acids and ammonia, is freed from the latter by concentrating *in vacuo*. Plimmer and Rosedale [1925] showed that, in concentrating the alkaline diamino-fraction at this stage, loss occurred, which was presumably due to decomposition of the amino-acids. The above procedure has however been employed, as any loss at this stage is not greater than that sustained by an earlier removal of the ammonia which would entail the formation of an additional precipitate and washing of a large bulk of barium sulphate. The aim is to carry through the operations of decomposing the phosphotungstates, filtering and concentrating as rapidly as possible. The excess of barium is now removed quantitatively by means of sulphuric acid and filtered off. The filtrate is then acidified to 1.5 % with sulphuric or acetic acid and histidine precipitated by adding a saturated solution of silver sulphate or acetate to the hot solution. After standing for 24 hours, the precipitate of the silver salt of histidine is filtered off and treated in accordance with the usual Kossel method.

A further quantity of silver salt is added to the filtrate which is made alkaline with baryta to secure precipitation of arginine. The filtrate from the silver-arginine compound is freed from silver by acidifying to 5 % with HCl and filtering. Lysine is precipitated from the filtrate by phosphotungstic acid and treated from this point in accordance with the Kossel method.

### RESULTS.

In order to compare the results of the Kossel and Van Slyke methods, the figures have all been reduced to the percentage of the total nitrogen which they represent. As already stated, during the Kossel procedure the amide- and humin-nitrogens were not estimated, so that these amounts have been secured only during the Van Slyke estimations. The average of the results obtained by Kossel's method are given in Table I.

Table I. *Results by the modified Kossel method.*

	Percentages of total nitrogen.				
	Pork muscle	Pig uterus	Human arm	Human foot	Breast carcinoma
Histidine-N	1.5	2.0	2.0	2.0	2.5
Arginine-N	7.0	13.0	5.0	7.0	7.0
Lysine-N	6.5	3.0	6.0	6.0	3.0
No. of exps.	3	5	4	4	3

While the above figures have been presented to the nearest 0.5 %, divergencies have occurred in some of the determinations composing the average. The most noteworthy concern arginine, where the figures varied in the "foot" experiments from 6.1 to 8.2 %, and in the "arm" experiments from 4 to 6.1 %. The most notable figure in the above table is that of lysine in carcinoma, but in this case the figures for the three determinations, 3.3, 3.8, 3.0 % show no very marked divergence from one another. As will be seen from Table II, the Van Slyke estimation gives a very low figure.

Table II. *Results by Van Slyke's method.*

Percentages of total nitrogen.										
		Diamino-N						Mono-amino-nitrogen		
		Anide-nitrogen	Humin-nitrogen	Total	Amino-nitrogen	Arginine-nitrogen	Histidine-nitrogen	Lysine-nitrogen	total	Total
Pig muscle	... ..	8.0	6.8	18.4	11.1	8.1	1.9	8.4	65.7	98.9
Pig uterus	... ..	8.5	8.7	18.0	9.6	8.3	3.2	6.5	64.2	99.4
Pig placenta	... ..	8.2	3.2	24.6	9.0	11.3	9.4	3.9	59.8	95.8
Pig foetus	... ..	7.5	14.8	15.4	8.8	8.5	0.3	6.6	58.0	95.7
Human arm	... ..	4.2	5.2	19.0	10.9	8.7	2.5	7.8	67.8	96.2
Human foot	... ..	4.4	5.0	18.3	9.9	9.0	2.5	6.8	68.1	95.8
Breast carcinoma	... ..	7.0	14.5	14.6	7.9	9.1	3.0	2.5	64.7	100.8
Chicken feathers	... ..	8.5	8.7	18.0	9.6	8.3	3.2	6.5	64.2	99.6
Maize-fed chicken flesh	...	8.5	8.7	13.4	7.6	8.7	2.6	2.1	66.8	97.4

In general, it will be seen that the Van Slyke method gives slightly higher results than the Kossel method. At the same time the difference if calculated in terms of the actual amino-acid is negligible. The conclusion may be drawn that normal muscle tissue does not vary to any extent in the various animals

or in different tissues of the same animal, provided the animal secures normal protein in the diet. The maize-fed chickens were taken from a group of experimental birds, which had received no protein other than that contained in maize. While maize contains other proteins than the deficient zein, it would appear that these are not present in sufficient quantity to produce normal flesh.

In Table III the amounts of the three diamino-acids in 100 g. of flesh are given.

Table III. *Diamino-acids, g. per 100 g. tissue.*

	Arginine	Histidine	Lysine
Pig muscle	1	0.27	1.7
Pig uterus	1	0.5	1.3
Pig placenta	1.2	1.1	0.9
Pig foetus	0.6	0.26	0.8
Human arm	0.75	0.25	0.8
Human foot	1	0.27	1
Breast carcinoma	0.63	0.24	0.3
Chicken feathers	3.71	1.7	4.9
Maize-fed chicken flesh	0.75	0.26	0.3

#### SUMMARY.

1. The diamino-acid content of several animal tissues has been determined by Van Slyke's method and by a modification of Kossel's method.
2. No fundamental differences occur in the two methods used.
3. It appears that the diamino-acid contents of the tissues of different animals, and of different parts of a normal animal are similar.
4. Low content of lysine has been found in carcinoma, and in chickens which have derived the whole of their food-protein from maize.

In conclusion, I desire to thank Prof. R. H. A. Plimmer most heartily for his guidance and advice throughout the course of this work, and also the Dean of this School and Dr M. J. Rowlands for the supply of material used.

#### REFERENCES.

- Plimmer (1916). *Biochem. J.* 10, 115.  
 — (1924). *Biochem. J.* 18, 105.  
 Plimmer and Lowndes (1927). *Biochem. J.* 21, 247.  
 Plimmer and Phillips (1924). *Biochem. J.* 18, 312.  
 Plimmer and Rosedale (1925). *Biochem. J.* 19, 1004.  
 Rosedale (1922). *Biochem. J.* 16, 27.



# CV. STUDIES ON COLOUR TESTS FOR STEROLS AND VITAMIN A.

## I. STEROL TESTS.

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*(Received May 9th, 1928.)*

NUMEROUS colour tests for sterols have been put forward during the last fifty years, the reagents employed including concentrated sulphuric acid [Salkowski, 1872], concentrated sulphuric acid with acetic anhydride [Liebermann, 1885], acetyl chloride with zinc chloride [Tschugaieff, 1900], arsenic and antimony trichlorides [Kahlenberg, 1922], and antimony pentachloride [Steinle and Kahlenberg, 1926]. In some cases the production of colour is activated by addition of benzoyl peroxide [Lifschutz, 1898], nitric acid [Whitby, 1923] or formaldehyde [Whitby, 1923]. The initial colours obtained are either blue or red, or a blend of these.

The first colour test for vitamin A, put forward by Drummond and Watson [1922], was based on the old Salkowski [1872] reaction which had long been used to estimate the "activity" of cod-liver oil. Many years before vitamins had been discovered, this characteristic reaction with concentrated sulphuric acid had been shown to be due to a constituent residing in the unsaponifiable fraction of the oil, and destroyed by oxidation when the oil became rancid. Other "vitamin" reagents (e.g. arsenic trichloride [Rosenheim and Drummond, 1925], antimony trichloride [Carr and Price, 1926]) would also appear to be closely related to sterol tests, and give similar, though more transient, colours.

In view of these facts, and of the gradually accumulating evidence in favour of vitamin A being a sterol derivative, it was thought of interest to make a comparative study of some of these colour tests put forward for sterols and for vitamin A.

## EXPERIMENTAL.

### *Sources of sterols employed.*

The cholesterol derivatives were prepared by Mr W. A. B. Sexton, working at Liverpool University, from pure cholesterol obtained from cod-liver oil. Feeding tests showed this cholesterol to be free from vitamins A and D. The purity of these derivatives, and the absence of ergosterol, was spectroscopically controlled. The ergosterol was obtained from ergot, and the sitosterol from wheat germ oil. Analytical data obtained on these sterols and derivatives are given in Table I.

*Reagents.*

These were usually prepared and applied according to the directions of their respective authors. In certain cases modifications were introduced on account of factors such as time, temperature, nature of solvent, concentration, presence of catalysts, which have been found to influence the results obtained.

Table I. *Sterols and sterol derivatives examined.*

Sterol or derivative	Formula	M. Pt.	No. of double bonds	$[\alpha]_D^{20}$
Cholesterol <sup>1</sup>	$C_{27}H_{48}OH$	148.5°	1	- 39.2°
Cholesteryl acetate	$C_{27}H_{46}COOCH_3$	114.5°	1	—
Cholesteryl chloride	$C_{27}H_{45}Cl$	96°	1	—
$\alpha$ -Cholesterylene	$C_{27}H_{44}$	78-79°	2	- 102.1°
Cholestene	$C_{27}H_{46}$	92°	1	- 53.05°
$\psi$ -Cholestene	$C_{27}H_{46}$	80°	1	+ 60.13°
Cholestylmethylxanthogenic ester	$C_{27}H_{48}OCSSCH_3$	127°	—	—
Cholestenone	$C_{27}H_{44}O$	80°	2	—
Dicholesteryl ether	$(C_{27}H_{45})_2O$	194-195°	2	—
Hydroxycholesterylene	$C_{27}H_{45}O$	110°	3	—
$\beta$ -Hydroxycholestenol acetate	$C_{27}H_{43}O_2COCH_3$	156°	—	—
Ergosterol	$C_{28}H_{48}O$	163°	3	- 127°
Sitosterol	$C_{27}H_{46}O$	132°	—	- 34.4°

<sup>1</sup> Freed from ergosterol by boiling in alcoholic solution with norite for several hours, and fractional crystallisation.

*Results.*

These may be summarised as follows.

1. Pure cholesterol, freed from ergosterol, gives with the "vitamin" reagents (concentrated sulphuric acid, arsenic and antimony trichlorides) red colours persisting for many hours. Similar results are obtained with cholesteryl acetate and chloride,  $\alpha$ -cholesterylene, cholestene and  $\psi$ -cholestene, but with the last two more time may be required for the colour to develop.

2. Cholesterol, cholesteryl acetate or chloride, cholestene and  $\psi$ -cholestene in chloroform solution, left in contact with concentrated sulphuric acid for some hours, and then diluted with more chloroform, give a purple or violet colour. Similar colours can be obtained by removal of the chloroform solution from the acid after less than a minute's contact, and addition to the former of a drop of formalin.

3. No colours are produced by formaldehyde alone with chloroform solutions of cholesterol or its derivatives which have not been in contact with concentrated sulphuric acid.

4. Irradiation of sterol derivatives generally has the effect of rendering the colours more transient. In the case of cholesterol, however, irradiation under certain conditions may develop the property of producing with the "vitamin" reagents blue colours, changing to red on standing for some hours.

5. Activation with other agents, such as acetic anhydride, benzoyl peroxide or formaldehyde, may lead to blue or purple colours being obtained on addition of the "vitamin" reagents.

6. Cholestenone, which has been suggested [Heilbron, Morton and Sexton, 1928] as being a typical product of irradiation, gives transient red colours with the "vitamin" reagents, and negative results (including red instead of blue with Liebermann's and Lifschutz's tests) with all other tests.

7. The other oxidation products of cholesterol which have been tested give negative results in all cases (dicholesteryl ether can be made to give red colours with modified Lifschutz's, Liebermann's, Tschugaieff's and "vitamin" tests).

8. Antimony pentachloride gives the colour sequence red  $\rightarrow$  blue  $\rightarrow$  red with all cholesterol derivatives examined except the oxidation products. Of these dicholesteryl ether, containing one proportion of oxygen, gives both red and blue, cholestenone and hydroxycholesterylene, containing two proportions of oxygen, give red only, and  $\beta$ -hydroxycholestenol acetate, containing four proportions of oxygen, gives negative results.

9. Introduction of sulphur into the side chain, as in cholesterylmethylxanthogenic ester, retards the development of the colours, but does not necessarily prevent it after liberation of sulphur compounds has taken place.

10. Ergosterol differs from cholesterol only in its results with the "vitamin" reagents. These give with ordinary concentrations of ergosterol the usual red, but if a higher initial concentration be employed (about 0.05 g. to 1 cc. of reagent) the red colour given by arsenic or antimony trichloride changes to purple or blue, on diluting with more reagent after a few moments.

11. Sitosterol gives similar results to cholesterol, but more slowly.

Details of the individual tests are given in Table II.

Table II. *Colour tests on sterols and derivatives.*

Sterol or derivative	"Vitamin" reagents						Whitby		Tschugaieff	Steinle and Kahlenberg
	Salkowski, Drummond and Watson	Rosenheim and Drummond	Carr and Price	Liebermann	Lifschutz <sup>1</sup>	Rosenheim (methylal and AsCl <sub>3</sub> )	A	B		
Cholesterol <sup>2</sup>	R	R	R	R $\rightarrow$ B	B $\rightarrow$ R	B $\rightarrow$ R	B	R*	R	R $\rightarrow$ B
Cholesteryl acetate	R	R	R	R $\rightarrow$ B	B $\rightarrow$ R	B $\rightarrow$ R	B	R	R	R $\rightarrow$ B
Cholesteryl chloride	R	R	R	R $\rightarrow$ B	B $\rightarrow$ R	B $\rightarrow$ R	B	R	R	R $\rightarrow$ B
$\alpha$ -Cholesterylene	R	R	R	R $\rightarrow$ B	P $\rightarrow$ R	B	B	R	R	R $\rightarrow$ P
Cholesterol, irradiated <sup>4</sup>	B $\rightarrow$ R*	B $\rightarrow$ R*	B $\rightarrow$ R*	R $\rightarrow$ B*	B $\rightarrow$ R*	B*	P	R	R	R $\rightarrow$ B
$\alpha$ -Cholesterylene, irradiated <sup>5</sup>	R	R	R	R $\rightarrow$ B	B $\rightarrow$ R	B	P	R	R	R $\rightarrow$ P
Cholestene	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	R $\rightarrow$ P	R	B	B	R	R	R $\rightarrow$ P
$\psi$ -Cholestene	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	R $\rightarrow$ P	R	B	B	R	R	R $\rightarrow$ P
Cholesterylmethylxanthogenic ester	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	R $\rightarrow$ P	$\rightarrow$ B	$\rightarrow$ R	P	$\rightarrow$ R	R	R $\rightarrow$ P
Cholestenone	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	R	R*	—	—	—	—	R
Dicholesteryl ether	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	$\rightarrow$ R	R*	—	—	—	R*	R $\rightarrow$ B
Hydroxycholesterylene	—	—	—	—	—	—	—	—	—	R
$\beta$ -Hydroxycholestenol acetate	—	—	—	—	—	—	—	—	—	—
Ergosterol	R	R <sup>3</sup>	R <sup>3</sup>	R $\rightarrow$ B	B	B	B	R	R	R $\rightarrow$ B
Sitosterol	R	R	R	R $\rightarrow$ B	B	B	P	R	R	R $\rightarrow$ B

<sup>1</sup> Heated in chloroform solution for a few hours at 37°, then cooled, and arsenic or antimony trichloride added.

<sup>2</sup> Freed from ergosterol as previously described.

<sup>3</sup> In high concentration the initial red colour changes rapidly to a blue which may persist for several hours.

<sup>4</sup> Irradiated half an hour at melting point.

<sup>5</sup> Irradiated until characteristic absorption bands disappeared.

"B" signifies a blue colour, "P" purple and "R" red.

$\rightarrow$  Indicates development of colour on standing.

\* Denotes that the colour is more transient.

## DISCUSSION.

In the case of colour tests put forward for vitamin A, it was first suggested by Rosenheim and Drummond [1925], and Takahashi *et al.* [1925], that the initial blue colour obtained, rather than the red colour which develops on ~~standing~~, is indicative of vitamin content. This initial blue colour has since been employed by a number of investigators to detect or estimate the vitamin in cod-liver oil and other sterol-containing natural products [Drummond, Channon and Coward, 1925; Carr and Price, 1926; Peacock, 1926; Rosenheim and Webster, 1926; 1927, 1, 2; Willimott, Moore and Wokes, 1926; Willimott and Wokes, 1927, 1, 2; Wokes and Willimott, 1927, 1, 2, 3]. Some interest, therefore, attaches to any case in which a sterol derivative produces with "vitamin" reagents blue colours similar to those attributed to the vitamin.

The results given above show that blue colours may be obtained from sterols under the following conditions:

- (1) with antimony pentachloride;
- (2) with acetic anhydride and concentrated sulphuric acid;
- (3) with "vitamin" reagents on ergosterol in high initial concentration;
- (4) with "vitamin" reagents on sterols treated with "oxidising" agents, formaldehyde or acetic anhydride.

It is true that these sterol colours are fairly stable, and may take a day or more to change to red or red-brown, whereas the "vitamin" colours are more transient, the blue colour in general having disappeared within a few minutes. But the above results show that irradiation of the sterols may cause the colours to be more transient, while it has been found possible, in the case of antimony trichloride on a physiologically tested cod-liver oil, to make the "vitamin" blue persist in measurable quantity for nearly an hour [Wokes and Willimott, 1927. 2].

Another characteristic property of the "vitamin" colours is their sequence, blue  $\rightarrow$  red. In the case of antimony pentachloride, of acetic anhydride and concentrated sulphuric acid, and of arsenic or antimony trichloride on ergosterol in high concentration, the initial colour obtained is red. The blue  $\rightarrow$  red sequence has, however, been obtained with cholesterol either irradiated at its melting point or oxidised under given conditions.

The case of ergosterol is exceptional in requiring a high initial concentration of sterol. Usually in sterol tests the most satisfactory concentration of sterol appears to be between 0.05 and 0.2 %. It has been shown by Wokes and Willimott [1927, 3] that the best results with antimony trichloride as a quantitative reagent for vitamin A in cod-liver oil are given by a concentration of oil of 1 to 5 %. Taking the unsaponifiable fraction of cod-liver oil as about 1 %, the optimum concentration of this fraction for "vitamin" tests would be from 0.01 to 0.05 %, or not very different from the optimum concentration for sterol tests.

Thus, of the four classes of blue colours given by sterols and their derivatives, it is those which are obtained by addition of "vitamin" reagents to cholesterol or certain of its derivatives or to ergosterol, after "oxidation" with benzoyl peroxide or nascent formaldehyde, which resemble most closely the "vitamin" colours. In regard to cholesterol itself (not purified from ergosterol) it was shown by Lifschutz many years ago [1908] that it gave with concentrated sulphuric acid, after treatment with benzoyl peroxide, a definite blue colour. Marston [1924] found that "oxycholesterol" prepared according to Lifschutz's method also gave a blue colour with arsenic trichloride, a reagent which had previously been shown by Kahlenberg [1922] to give a red colour with untreated cholesterol. Robertson [1925] came to the conclusion that when cholesterol is oxidised by Lifschutz's method the composition of the product varies, and found it more satisfactory to aerate in aqueous colloidal suspension at 100° in presence of an acetone extract of brain tissue. Rosenheim [1927] pointed out that cholesterol or ergosterol, after treatment with either benzoyl peroxide or nascent formaldehyde (obtained from methylal) will give blue colours with arsenic or antimony trichloride. Moore and Willimott [1927] showed that cholesterol, after heating in air to about 150° for half to two hours will give ultramarine colours, persisting some hours, with antimony trichloride. The same colours were obtained from cholesterol which had been heated in colloidal aqueous suspension at 90° for an hour or more, the presence of brain tissue extract not being found essential.

Turning to cholesterol derivatives, the new results here recorded may, perhaps, permit certain deductions to be drawn. Whitby [1923] suggested that in a typical sterol colour test the first stage consisted in the production of a colourless hydrocarbon, such as cholesterylene, by the action on cholesterol of the condensing agent (*e.g.* concentrated sulphuric acid), and that this product then coupled with a second substance (*e.g.* formaldehyde) to give the coloured product. But the writer has failed to obtain any colours with cholesterylene or cholestene when treated with formaldehyde only (either as formalin solution or produced in the nascent condition by warming a methylal solution of the sterol with dilute acid). This would seem to be evidence against Whitby's theory. The only noticeable result of the loss of the hydroxyl group was a distinct retardation in the rate of development of the colour in the case of cholestene and  $\psi$ -cholestene, but not in the case of cholesterylene, which contains an additional double bond. Variation in the position of the double bond, or replacement of the hydroxyl group by an acid radicle (acetate or chloride) made no appreciable difference to the colours obtained.

The most interesting results have been obtained with "vitamin" reagents on cholesterol which has either been irradiated or treated with oxidising or other agents, such as benzoyl peroxide, formaldehyde and acetic anhydride. From the recent work of Heilbron, Morton and Sexton [1928] the inference

might be drawn that when cholesterol is irradiated, dehydrogenation takes place, with formation of oxidation products similar to cholestenone. Tests applied by the writer to the latter compound have shown it to give with the "vitamin" reagents rather unstable red colours only, in contradistinction to initial blue colours obtained from irradiated cholesterol. A failure to obtain blue colours was also experienced after irradiation of cholesterylene and certain other cholesterol derivatives. These results do not necessarily imply that cholestenone is not a product of the irradiation of cholesterol, but there seems to be a strong possibility that, under suitable conditions, there is formed some other chromogen which is responsible for the initial blue colour. Further oxidation may lead to formation of chromogens giving red colours only, and finally to completely negative results. Oxidation of cholesterol can be made to give mixed products which when treated with "vitamin" reagents yield initial blue colours, changing gradually to red, which very closely resemble the "vitamin" colours given by the same reagents on cod-liver oil and other natural sources of vitamin A. But it has not yet been possible to isolate from these oxidation mixtures pure substances giving initial blue colours with the vitamin reagents, and only red chromogens have so far been obtained. With sterols as with the "vitamin" colours, the blue chromogen is unstable, and is destroyed by attempts to isolate it.

The writer is indebted to Prof. Heilbron for samples of cholesterol derivatives, and to Messrs Burroughs Wellcome for a specimen of sitosterol.

## REFERENCES.

- Carr and Price (1926). *Biochem. J.* 20, 497.  
Drummond and Watson (1922). *Analyst*, 47, 341.  
Drummond, Channon and Coward (1925). *Biochem. J.* 19, 1047.  
Heilbron, Morton and Sexton (1928). *J. Chem. Soc.* 47.  
Kahlenberg (1922). *J. Biol. Chem.* 52, 217.  
Liebermann (1885). *Ber. deutsch. chem. Ges.* 18, 1804.  
Lifschutz (1898). *Z. Nahr. Genussm.* 1, 21.  
— (1908). *Ber. deutsch. chem. Ges.* 41, 252.  
Marston (1924). *Austral. J. Exp. Biol. Med. Sci.* 1, 53.  
Moore and Willimott (1927). *Biochem. J.* 21, 585.  
Peacock (1926). *Lancet*, i, 328.  
Robertson (1925). *Austral. J. Exp. Biol. Med. Sci.* 2, 83.  
Rosenheim (1927). *Biochem. J.* 21, 386.  
Rosenheim and Drummond (1925). *Biochem. J.* 19, 753.  
Rosenheim and Webster (1926). *Biochem. J.* 20, 1342.  
— (1927, 1). *Biochem. J.* 21, 111.  
— (1927, 2). *Biochem. J.* 21, 389.  
Salkowski (1872). *Z. anal. Chem.* 11, 443.  
Steinle and Kahlenberg (1926). *J. Biol. Chem.* 67, 425.  
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Inst. Phys. Chem. Res., Tokyo*, 3, 81.  
Tschugaieff (1900). *Chem. Ztg.* 24, 542.  
Whitby (1923). *Biochem. J.* 17, 5.  
Willimott, Moore and Wokes (1926). *Biochem. J.* 20, 1292.  
Willimott and Wokes (1927, 1). *Pharm. J.* 118, 217.  
— (1927, 2). *Lancet*, ii, 8.  
Wokes and Willimott (1927, 1). *Biochem. J.* 21, 419.  
— (1927, 2). *Pharm. J.* 1, 752.  
— (1927, 3). *Analyst*, 52, 515.

# CVI. THE EFFECT OF INANITION AND VITAMIN B DEFICIENCY ON THE ADRENAL GLANDS OF THE PIGEON.

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(Received May 10th, 1928.)

## INTRODUCTION.

IN a recent communication [Marrian *et al.*, 1927] it was shown that the hypertrophy of the adrenal glands which can be produced in pigeons by allowing them to feed voluntarily on a diet deficient in vitamin B, could also be produced by inanition even when liberal supplies of extracts rich in the vitamin were fed. It was at first thought that this indicated that the adrenal hypertrophy of vitamin B deficiency was entirely due to the state of inanition that almost invariably accompanies this condition. However, they found that when the pigeons were forcibly fed with the deficient diet, so that the loss of body weight at the conclusion of the experiment was negligible, the adrenals were enlarged to approximately the same extent.

The present work was undertaken in order to confirm and if possible find some explanation for these curious results. In addition it was thought desirable to investigate the possibility of a deficiency of one only of the two recently recognised factors in the vitamin B complex being involved in the causation of this hypertrophy.

## EXPERIMENTAL.

The observations reported here were carried out on the same series of pigeons upon which a study of the effect of vitamin B deficiency and inanition on the testis was carried out [Marrian and Parkes, 1928].

Male birds were exclusively used, since the size of the adrenal glands seems to vary appreciably with sex, and in the case of the hen bird there would appear to be a definite hypertrophy during ovulation [Riddle, 1922].

The birds were then divided into five groups which were arranged and treated in the following manner:

Group	Diet	Vitamin preparations given	Object of experiment
A	20 g. vitamin B-deficient diet daily forcibly fed	1 g. yeast extract daily	Normal controls
B	" "	—	Vitamin B deficiency
C	" "	1 g. autoclaved yeast extract daily	Vitamin B <sub>1</sub> deficiency
H	" "	Peters's torulin curative dose x 3	Vitamin B <sub>2</sub> deficiency
SM	Starving	1 g. yeast extract daily	Inanition

The deficient diet used was an artificial one, of the type used by Randoin and Simmonet [1924]. The method of its preparation and the technique of forcible feeding have been described fully elsewhere [Kon and Drummond, 1927; Marrian *et al.*, 1927].

The vitamin preparations were administered to the birds in aqueous solution directly into the crop through a rubber catheter tube. The autoclaved yeast extract (known to contain no vitamin B<sub>1</sub>) was prepared by heating a slightly alkaline solution of commercial yeast extract to 110° for 3 hours. Before use this was carefully neutralised. The preparation containing vitamin B<sub>1</sub> only was prepared according to the method of Kinnersley and Peters [1927].

In order to avoid errors in body weight due to overloaded crops, the birds were all starved for 24 hours before the initial weighings were made. At the conclusion of the experiments any food remaining in the crop was quantitatively removed, dried and weighed. The final weight of each bird was obtained by subtracting this figure from the total body weight immediately before death.

The vitamin B<sub>1</sub>-deficient pigeons (groups *B* and *C*) were killed at the first signs of typical polyneuritis, while those in groups *A* and *H*, which would not be expected to show terminal symptoms were killed as soon after as possible. The starving pigeons were killed at the beginning of the final collapse, which has been described by Marrian *et al.* [1927].

Immediately after the death of the bird the adrenal glands were cut out, carefully dissected from any adhering connective tissue and fat, and weighed. Adrenaline was estimated in some of these by the modified Folin, Cannon and Denis method suggested by Baker and Marrian [1927]. Estimations of the moisture content of a number of others were carried out to investigate the possibility of the observed hypertrophy being to some extent due to oedema.

#### *General behaviour of the birds.*

A brief description only of the general behaviour of this series of pigeons will be made, since this has been dealt with elsewhere [Marrian and Parkes, 1928].

With one exception, which will be referred to in a later section of the paper, these birds behaved in a normal manner and in all cases the body weights were satisfactorily maintained. The majority of the birds in groups *B* and *C* showed the classical symptoms of neck retraction, but in a few instances the condition called emprosthotonus by McCarrison [1919] was observed. The group *H* birds, like those in groups *B* and *C*, vomited daily to some extent, but otherwise no definite symptoms could be observed.

Since the body weights of nearly all the birds in these groups were satisfactorily maintained, it is evident that the greater part of the diet was retained and satisfactorily metabolised.



The general condition of the pigeon during inanition has been fully discussed previously [Marrian *et al.*, 1927].

Details of the weight changes, symptoms, and times on the diets of the birds are shown in Table I.

*Weight of the adrenal glands.*

On referring to Table I it will be seen that the adrenals of the pigeons that were forcibly fed on the deficient diet and also those of the starving birds receiving vitamin B are definitely hypertrophied when compared with those of the forcibly fed controls in group A. This confirms the findings of Marrian *et al.* [1927].

The results from the birds in groups C and H, deficient in vitamins B<sub>1</sub> and B<sub>2</sub> respectively, are however not quite so clear cut. The average figure for the weight of the glands of the group C birds seems to indicate that the hypertrophy is of almost the same order as in group B. The weight of the adrenals of C 66 however was more than twice the average of the remainder in the group, and since this bird was abnormally oedematous it would almost seem justifiable to eliminate this result. If this is done, an average figure for the adrenal weight in group C of 0.0425 g. is obtained, which, although quite definitely greater than the control, is considerably less than the group B average.

The average weight of the adrenals of the group H pigeons is quite clearly significantly less than the group B average, but still definitely above that of the control group. The possibility must be borne in mind that these birds were vomiting an appreciable quantity of the antineuritic vitamin and that in consequence they were suffering from a mild vitamin B<sub>1</sub> deficiency. This however seems unlikely since the birds were receiving three times the normal curative dose daily by mouth and in addition were injected with the extract on three occasions.

The author is inclined to the view that this slight adrenal hypertrophy of the pigeons deficient in the thermostable factor is quite definite and also that the difference in degree of hypertrophy between groups B and C is real and significant. Thus it would appear that a deficiency of each of the two factors plays a part in the production of the adrenal enlargement observed in vitamin B deficiency, the vitamin B<sub>1</sub> deficiency being the more important.

This conclusion is in agreement with the recent observations of Findlay [1928] who has shown that the adrenal glands of rats are enlarged as a result of vitamin B<sub>1</sub> or B<sub>2</sub> deficiency. The greater degree of hypertrophy of the adrenals of Findlay's rats deficient in vitamin B<sub>2</sub> only, when compared with the group H pigeons, may be explained by the fact that they were fed on the deficient diet for over twice as long as those deficient in vitamin B<sub>1</sub>, whereas the pigeons in groups C and H were receiving the deficient diets for approximately the same length of time.

Another possible explanation of these results is suggested by the recent

work of Williams and Waterman [1927] who on rather scanty evidence suggest that a third factor (present in whole wheat) is necessary for the satisfactory health and growth of the pigeon, vitamin B<sub>2</sub> being relatively unimportant. Assuming that it is present in yeast extract, it is conceivable that the adrenal hypertrophy might in some degree be due to a deficiency in this factor.

Table I.

Group	No.	Initial weight (g.)	Final weight (g.)	Weight change %	Days on diet	Weight adrenals (g.)	Total adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Moisture content of adrenals %	Remarks on condition of bird when killed
A	5	405	475	+19.7	18	0.0232	0.057	2.46	—	—
A	3	389	476	+23.4	25	0.0270	0.068	2.52	—	—
A	6	292	348	+19.1	26	0.0277	0.062	2.24	—	—
A	39	448	500	+12.1	22	0.0174	—	—	70.7	—
A	43	406	452	+11.8	22	0.0287	0.060	2.09	—	—
A	42	471	475	+ 0.8	23	0.0352	—	—	71.8	—
A	40	434	443	+ 2.1	24	0.0294	—	—	74.7	—
A	38	421	440	+ 4.3	25	0.0268	—	—	72.0	—
A	44	348	420	+17.6	25	0.0248	—	—	70.5	—
A	37	411	390	- 5.1	26	0.0297	—	—	73.3	—
A Average		402	442	+10.4	24	0.0270	0.062	2.33	72.1	—
B	7	394	398	+ 1.0	17	0.0539	0.066	1.22	—	Severe neck retraction
B	9	382	352	- 7.8	18	0.0613	0.172	2.12	—	Neck retraction
B	8	401	434	*	18	0.0696	0.078	1.12	—	Neck retraction. Breast very oedematous
B	11	350	354	+ 1.1	21	0.0610	0.136	1.17	—	Emprosthotonus
B	10	343	279	-18.6	22	0.0515	0.063	1.22	—	Neck retraction
B	52	423	411	- 2.8	16	0.0586	—	—	75.2	Weak. Slight neck retraction
B	45	406	393	- 3.2	17	0.0573	—	—	76.1	Severe neck retraction
B	51	516	456	-11.6	19	0.1062	—	—	75.9	Neck retraction
B	50	431	355	-17.6	19	0.1023	—	—	75.6	Emprosthotonus
B	48	424	350	-17.4	24	0.0424	—	—	75.5	Severe neck retraction
B Average		407	378	- 7.7	19	0.0704	0.103	1.37	75.7	—
C	18	394	386	- 2.0	22	0.0376	0.051	1.36	—	Neck retraction
C	57	348	368	+ 5.7	16	0.0441	0.045	1.02	—	Emprosthotonus
C	59	439	444	+ 1.0	22	0.0677	0.051	0.74	—	Emprosthotonus
C	66	427	519	*	15	0.1222	0.122	1.00	—	Neck retraction. Breast extremely oedematous. Abdominal cavity full of fluid
C	61	320	325	+ 1.6	16	0.0294	0.036	1.29	—	Emprosthotonus
C	64	435	481	+10.5	18	0.0399	0.102	2.55	—	Severe neck retraction
C	65	430	419	- 2.5	20	0.0360	0.062	1.72	—	Neck retraction
C Average		399	420	+ 2.4	19	0.0536†	0.067	1.38	—	—
H	70	372	324	-12.8	23	0.0508	0.084	1.65	—	—
H	68	395	407	+ 2.9	23	0.0282	0.063	2.23	—	—
H	69	345	320	- 7.2	23	0.0330	0.073	2.21	—	—
H	72	441	374	-15.0	23	0.0845	0.105	3.03	—	—
H	67	371	336	- 9.4	23	0.0343	0.091	2.65	—	—
H Average		365	352	- 8.3	23	0.0361	0.063	2.35	—	—
SM	75	312	204	-34.6	11	0.0358	0.088	2.46	—	—
SM	73	324	302	-37.7	17	0.0596	0.122	2.32	—	—
SM	74	287	237	-36.8	17	0.0533	—	—	79.2	—
SM	76	435	273	-37.2	21	0.0323	—	—	77.2	—
SM	78	478	285	-40.4	22	0.0326	0.137	2.59	—	—
SM Average		337	240	-37.7	18	0.0494	0.116	2.46	78.2	—

\* Both these birds were extremely oedematous, hence the body weight changes are of little significance.

† 0.0425, excluding pigeon C 66.

*Oedema of the adrenal glands.*

A number of determinations of the moisture content of the adrenals of the groups *A*, *B* and *SM* birds were carried out in order to see to what extent the adrenal enlargement in the two latter groups could be accounted for by an increase in the water content of the glands. Table I clearly shows that in both cases the glands were quite distinctly oedematous.

In the case of the starving birds this oedema was so marked as to account for nearly half of the total observed hypertrophy.

The following table summarises these results.

Table II.

Group	Fresh weight adrenals g.	Dry weight adrenals g.	Water %	Hyper- trophy calculated on fresh weight %	Hyper- trophy calculated on dry weight %	Proportion of hyper- trophy due to oedema %
<i>A</i> (av. of 6)	0.0272	0.0075	72.1	—	—	—
<i>B</i> (av. of 5)	0.0734	0.0178	75.7	169	137	19
<i>SM</i> (av. of 2)	0.0528	0.0115	78.2	94	53	44

*The nature of the adrenal hypertrophy.*

Several observers seem to be agreed that during inanition there is an increase in adrenaline roughly corresponding to the enlargement of the gland in the pigeon [McCarrison, 1919, 1920; Marrian *et al.*, 1927]. The three adrenaline estimations carried out on the *SM* group of pigeons seem to confirm these observations, although the experiments are admittedly too few in number. So far as the author is aware, no histological study has yet been made of the adrenal glands of starving pigeons, so that it is as yet impossible to say definitely whether this represents an actual proliferation of the medullary cells, a hyperactivity of the medulla which remains constant in size, or a decreased output of adrenaline. As a result of studies carried out on pigeons fed on polished rice, Kellaway [1921] explains the increased adrenaline content of the hypertrophied glands by postulating that the adrenaline output is decreased by reason of the lowered metabolism of the bird. This however seems to be unlikely since at the time when the enlarged gland shows a high adrenaline content, the cloacal temperature of the bird is not necessarily markedly subnormal. In the case of the adult rat Vincent and Hollenberg [1921] showed that the adrenaline content of the adrenals is increased after 2-3 days' starvation, and at this early stage of inanition the body temperature and metabolism of the rat are not greatly reduced [Drummond and Marrian, 1926]. McCarrison [1920] regards the increased adrenaline content of the glands as representing true medullary hypertrophy. This is regarded as being the more acceptable hypothesis.

A discussion of the nature of the hypertrophy resulting from vitamin B deficiency is rendered difficult owing to the facts that many of the birds

examined by previous workers have been virtually starving at the time of incidence of the nervous symptoms and that much of the evidence is apparently conflicting. Thus McCarrison [1919] examined starving pigeons that had lost an average of 23 % of their body weight, while his polished rice birds had lost about 30 %. It is not surprising therefore that these birds gave a similar series of results to his starving ones. Kellaway [1921] made a study of pigeons fed voluntarily and forcibly on polished rice. In both cases he showed that there was a marked increase in the adrenaline content of the glands. His forcibly fed birds, however, although showing a loss of body weight (15 %) were far from being in a state of inanition. Beznak [1923] on the other hand, could find no increased amount of adrenaline in the hypertrophied glands of polyneuritic pigeons that had lost as much as 22 % of their original body weight.

The histological evidence suggests that it is the cortex that is mainly involved in the hypertrophy [Findlay, 1921; Lasowsky and Sumnitzki, 1926]. Findlay indeed showed that after curing polyneuritic pigeons with yeast, there occurred a rapid decrease in the cortical lipoids. It seems fairly clear therefore that in experimental beriberi changes do occur in the adrenal cortex.

Marrian *et al.* [1927] showed that in five forcibly fed vitamin B-deficient pigeons, whose average loss of body weight was only 5 %, the adrenals were considerably hypertrophied while the adrenaline content was not increased. It was pointed out that this might be explained on the assumption that the cortex alone hypertrophied when starvation did not complicate the picture.

However in the five group B pigeons that were examined, two cases (B 9, B 11) were found where the adrenals contained an abnormally large amount of adrenaline. The other three cases were entirely in line with the results of Marrian, Baker, Drummond and Woollard. It seems to be necessary therefore to conclude that in certain cases of experimental beriberi where the adrenaline content of the glands is increased, even when the starvation factor is eliminated, the medulla may be involved in the hypertrophy to some extent.

In spite of the somewhat disturbing nature of these results the bulk of the evidence certainly seems to indicate that the cortex is the site of the adrenal hypertrophy in most cases of vitamin B deficiency, the medulla being involved when starvation complicates the picture and also in certain other random cases.

This hypothesis receives some support from recent work on the function of the adrenal cortex. Evidence based on observations on adrenalectomised animals is accumulating to show that the adrenal cortex has the function of removing or neutralising in some manner various toxins, bacterial or otherwise, in the body [Lewis, 1923; Jaffe, 1926; Banting and Gairns, 1926; Belding and Wyman, 1926; Torino and Lewis, 1927], although this theory is strenuously denied by Stewart, Rogoff and their collaborators in a series of papers.

It also seems to be an established fact that bacterial infections and certain types of intoxication may cause an enlargement of the adrenal cortex.

It is interesting to note that McCarrison [1919] showed that in polyneuritic pigeons that were also suffering from various bacterial infections the adrenals were considerably enlarged without any corresponding increase in the amount of adrenaline. Since the symptoms in such birds were observed at an earlier date than in uninfected ones, one might expect that the starvation factor would be decreased or altogether eliminated. Thus the hypertrophy might be due either to vitamin B deficiency, or to the bacterial toxin, or more probably to both.

*Origin of the nervous symptoms arising from vitamin B deficiency.*

The view has often been expressed that the underlying cause of the nervous symptoms observed in vitamin B deficiency is a toxic substance. Thus one might build up an attractive theory explaining the adrenal hypertrophy in beriberi as an attempt by the body to eliminate the "beriberi" toxin. It should be possible to test this theory by showing that adrenalectomised animals develop beriberi more rapidly than do normal animals. Several unsuccessful attempts have been made by the author to do this. A few initial experiments were carried out on rats and in one instance convulsive seizures were observed a few days after the animal had been placed on the deficient diet. There were no grounds to believe however that these convulsions were specifically due to the vitamin deficiency, since convulsions are often a symptom of adrenal insufficiency in normally fed animals. In addition it has been shown by the author that adrenalectomised rats survive starvation for a much smaller period than normal rats (unpublished work), and since marked loss of appetite is a symptom both of adrenal insufficiency and vitamin B deficiency, it seemed to be useless to continue such experiments.

*Beriberi-like symptoms in normal pigeons.*

An extraordinary case was observed among the group A pigeons, which were receiving 1 g. of yeast extract daily. On the 19th and 20th days it was noticed that pigeon A 4 was far from well. After each meal there was considerable vomiting and in general it may be said that the condition of the bird closely resembled that of the vitamin B-deficient pigeons just before the incidence of the nervous symptoms. On the morning of the 21st day this bird was found in a condition absolutely indistinguishable from the usual symptoms of experimental "beriberi." The head of the bird was severely and persistently retracted and there was a periodic tendency to turn "cart-wheels." It would be no exaggeration to say that this bird seemed to be a more characteristic case of "beriberi" than many of those fed on deficient diets. The initial weight of the pigeon was 389 g. At the time that the symptoms appeared the total body weight was 433 g. The weight of food in

the crop was judged to be no more than 30 g., so evidently no loss of body weight had occurred.

It appeared extremely unlikely that this curious condition could be caused by a vitamin deficiency resulting from the vomiting of the administered yeast extract since no vomiting had been noticed until the 19th day. Even if the whole of the yeast extract had been expelled on the 19th and 20th days, it is quite inconceivable that this short period of deficiency could be the cause of the symptoms.

After some hours, as the condition remained unchanged, it was decided to see if the condition was curable by an injection of antineuritic vitamin. Accordingly 1 cc. of an extract made by the method of Kinnersley and Peters, that was known to be curative in doses of 0.5 cc., was injected. After 3 hours the pigeon appeared to be no better. The following morning the symptoms were still severe although the bird appeared considerably weaker. It was quite evident therefore that this condition was not curable by vitamin B<sub>1</sub>. The bird was subsequently killed while still showing these symptoms.

This case goes to strengthen the belief, which is rapidly growing among workers on beriberi in the pigeon, that the symptom of neck retraction is not specific to vitamin B deficiency and in fact may occur as a result of a variety of causes. Thus Marrian *et al.* [1927] observed symptoms of neck retraction in starving pigeons that were receiving ample supplies of yeast extract, and Williams [1927] reports a similar condition in pigeons that were fed exclusively on rice polishings. The author has also observed on one occasion well-defined neck retraction in a normal pigeon that was partially anaesthetised with ether.

#### SUMMARY.

1. The results obtained by Marrian, Baker, Drummond and Woollard showing that adrenal hypertrophy occurred in starving pigeons receiving vitamin B and also in pigeons forcibly fed on an artificial vitamin B-free diet to exclude the starvation factor, have been confirmed.
2. The hypertrophy occurring in the vitamin B-deficient pigeons seems to be mainly due to a deficiency in vitamin B<sub>1</sub>, but it is considered that a deficiency of vitamin B<sub>2</sub> is also a contributory factor.
3. The adrenal glands of both the starving and the vitamin B-deficient pigeons are oedematous. This oedema accounts for 19 % of the total hypertrophy shown by the vitamin B-deficient birds and 44 % in the case of the starving pigeons.
4. The question whether the adrenal hypertrophy occurring in starvation and vitamin B deficiency is medullary or cortical is discussed. It is considered possible that in starvation the changes occur mainly in the medulla while in vitamin B deficiency the cortex is mainly concerned. In certain cases changes in the medulla may occur in vitamin B deficiency.

5. A brief reference to a pigeon, forcibly fed on a complete diet, that developed acute symptoms resembling those due to vitamin B deficiency is made. This condition, although identical in appearance with avian beriberi, was not curable by vitamin B<sub>1</sub>.

The author is deeply indebted to Prof. J. C. Drummond for continual help and advice, also to Prof. Bertrand for the hospitality of his laboratory during part of this work.

## REFERENCES.

- Baker and Marrian (1927). *Biochem. J.* **21**, 1005.  
Banting and Gairns (1926). *Amer. J. Physiol.* **77**, 100.  
Belding and Wyman (1926). *Amer. J. Physiol.* **78**, 50.  
Beznak (1923). *Biochem. Z.* **141**, 1.  
Drummond and Marrian (1926). *Biochem. J.* **20**, 1229.  
Findlay (1921). *J. Path. Bact.* **24**, 175.  
—— (1928). *J. Path. Bact.* **31**, 353.  
Jaffe (1926). *Amer. J. Path.* **11**, 421.  
Kellaway (1921). *Proc. Roy. Soc. Lond. B.* **92**, 6.  
Kinnersley and Peters (1927). *Biochem. J.* **21**, 777.  
Kon and Drummond (1927). *Biochem. J.* **21**, 632.  
Lasowsky and Sumnitzki (1926). *Arch. path. Anat. Physiol.* **262**, 101.  
Lewis (1923). *Amer. J. Physiol.* **64**, 512.  
Marrian, Baker, Drummond and Woollard (1927). *Biochem. J.* **21**, 1336.  
McCarrison (1919). *Ind. J. Med. Res.* **6**, 275.  
—— (1920). *Proc. Roy. Soc. Lond. B.* **91**, 103.  
Marrian and Parkes (1928). *J. Roy. Micr. Soc.* (In press.)  
Randoin and Simmonet (1924). *Bull. Soc. Chim. Biol.* **6**, 601.  
Riddle (1922). *Proc. Soc. Exp. Biol. Med.* **19**, 280.  
Torino and Lewis (1927). *Amer. J. Physiol.* **81**, 405.  
Vincent and Hollenberg (1921). *J. Physiol.* **54**, lxxix.  
Williams (1927). *Biochem. J.* **21**, 1349.  
Williams and Waterman (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 1.

# CVII. DETERMINATION OF HYDROGEN ION CONCENTRATIONS IN PHOSPHATE AND BORATE MIXTURES BY MEANS OF THE QUINHYDRONE ELECTRODES.

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(Received May 10th, 1928.)

## I. INTRODUCTION.

THE application of the quinhydrone electrode (or the quinoquinhydrone electrode or the hydroquinhydrone electrode) as substitute for the hydrogen electrode for the determination of  $p_H$  is based on the fact that the values of the potentials between one of these electrodes and the hydrogen electrode vary but very little if the electrolytes of the two electrodes of the chain examined are identical. For aqueous electrolytes at 18° the following values as a rule may be used:

quinhydrone-hydrogen	...	...	...	0.7042 volt
quinoquinhydrone-hydrogen	...	...	...	0.7562 „
hydroquinhydrone-hydrogen	...	...	...	0.6176 „

The potential shown above of the simple quinhydrone electrode being only valid for dilute solutions (up to 0.1–0.2 *M*), the constants of the quinoquinhydrone and hydroquinhydrone electrodes should also be valid at higher concentrations, and this has been verified by means of experiments [Biilmann, 1921, 1927; Biilmann and Lund, 1923].

As these electrodes are often applied in biological researches, it seems important to know how they act in phosphate and borate mixtures, and researches on these solvents have only been made with a simple quinhydrone electrode.

In the following we shall first give the determinations of the fundamental potentials which may be used for the application of the different quinhydrone electrodes in phosphate and borate solutions. The solutions examined are:

### *Phosphate mixtures.*

1/15 <i>M</i> $KH_2PO_4$	1/15 <i>M</i> $Na_2HPO_4$	$p_H$
cc.	cc.	
900	100	5.90
700	300	6.45
500	500	6.81
300	700	7.16
200	800	7.38
100	900	7.73
50	950	8.04



*Borate mixtures.*

$\frac{1}{5} M H_2BO_3$ $\frac{1}{20} M NaCl$	$\frac{1}{20} M Na_2B_4O_7$	$p_H$
cc.	cc.	
900	100	7.34
800	200	7.78
700	300	8.05

Further, we have examined some phosphate and bicarbonate solutions saturated with carbon dioxide, and finally we shall investigate the influence of these new determinations on the results of the researches which Biilmann and Katagiri [1927] and Grossman [1927] have recently published in this *Journal*. Before dealing with the experiments, it must be remembered that in the simple quinhydrone electrode the solution examined is saturated with quinhydrone, in the quinoquinhydrone electrode with quinone and quinhydrone and in the hydroquinhydrone electrode with quinol and quinhydrone. Whereas quinhydrone is not very soluble in water (about  $0.02 M$ ) and the saturated solution of quinone in water is only about  $0.1 M$ , the saturated solution of quinol is about  $0.5 M$  at  $18^\circ$  [Biilmann, 1927; Biilmann and Lund, 1923].

## II. THE QUINHYDRONE ELECTRODE.

The potential of the quinhydrone electrode compared with the hydrogen electrode in the same electrolyte is generally assumed to be  $0.7042$  volt at  $18^\circ$ . This value gives very good determinations of  $p_H$  in many dilute solutions of acids or mixtures of acids and salts. Now we have examined the quinhydrone electrode in the phosphate mixtures mentioned above and found the values recorded in Table I.

Table I.

		+ Quinhydrone, phosph. mixt.   phosph. mixt., $H_2$ --. $18^\circ$ .					
$p_H$	5.90	6.45	6.81	7.16	7.38	7.73	8.04
$\pi$	0.7060	0.7057	0.7056	0.7058	0.7058	(0.7062)	(0.7072)
Mean value: 0.7058.							

At  $p_H$  7.73 and 8.04 the potentials are not so stable as in the more acid solutions. The measurements show that when  $p_H$  in phosphate mixtures is determined with the quinhydrone electrode, the value  $0.7058$  ought to be used for the calculation instead of  $0.7042$  which will give  $p_H$  values about  $0.03$  too low. When measuring the borate mixtures at  $18^\circ$ , the values given in Table II were obtained.

Table II.

		+ Quinhydrone, borate mixt.   borate mixt., $H_2$ --. $18^\circ$ .	
$p_H$		7.78	8.05
$\pi$		0.7049	0.7046
Mean value: 0.7045.			

The mean value  $0.7045$  agrees so well with  $0.7042$  that the difference only amounts to  $0.005$  in the  $p_H$  value.

## III. THE QUINOQUINHYDRONE ELECTRODE.

It is to be remembered that the quinoquinhydrone electrode is saturated both with quinone and with quinhydrone. In phosphate mixtures at 18° the values recorded in Table III were found.

Table III.

	+ Quinone, quinhydrone, phosph. mixt.   phosph. mixt. $H_2$ - . 18°.						
$p_H$	5.90	6.45	6.81	7.16	7.38	7.73	8.04
$\pi$	0.7562	0.7560	0.7561	0.7562	(0.7565)	(0.7565)	(0.7530)

At  $p_H$  7.38, 7.73 and 8.04 the potentials drifted so rapidly that exact determinations could not be obtained, and on the whole the determinations with these electrodes had to be made soon after mounting the electrodes. The mean of the first four potentials, 0.7561 volt, agrees well with the values found by Billmann and Lund [1923] in hydrochloric acid and sulphuric acid (0.7561–0.7564, mean 0.7562) and with the value 0.7564 found by Schreiner [1925] in 2 *N* sulphuric acid.

In borate mixtures  $p_H$  7.34–8.05 the potentials drifted so rapidly that exact readings could not be taken.

## IV. THE HYDROQUINHYDRONE ELECTRODE.

In the hydroquinhydrone electrode the solution is saturated both with quinol (hydroquinone) and with quinhydrone. The potentials compared with those of the hydrogen electrode in phosphate mixtures are shown in Table IV.

Table IV.

	+ Quinol, quinhydrone, phosph. mixt.   phosph. mixt., $H_2$ - . 18°.						
$p_H$	5.90	6.45	6.81	7.16	7.38	7.73	8.04
$\pi$	0.6229	0.6221	0.6229	0.6238	0.6253	0.6304	0.6411

From the table we see that  $\pi$  is not constant, but increases considerably when the  $p_H$  of the solution examined increases from 5.90 to 8.04. This is not due to an influence of the phosphate mixtures on the activities of quinone and quinol, as these are constant in the saturated solutions. Accordingly, it is to be assumed that the quinol exercises an influence on the phosphate mixtures changing their  $p_H$  values increasingly with increasing  $p_H$  values, which gives rise to corresponding changes of the potentials. An influence of this kind may *a priori* be assumed for the basic solutions, as quinol is a weak acid, but it will be seen that the acid effect of the quinol does not alone account for the observed changes in  $p_H$ . An influence of quinol on the activity of the ions may, therefore, also take place.

In borate mixtures we found an influence similar to that in phosphate mixtures (Table V), but later we shall see that in this case we have only or chiefly to do with an acid effect of the quinol.

Table V.

	+ Quinol, quinhydrone, borate mixt.   borate mixt., $H_2$ - . 18°.		
$p_H$	7.34	7.76	8.05
$\pi$	0.6264	0.6277	0.6296

In order to determine the influence of quinol on the  $p_H$  value of a phosphate mixture we first tried to measure the potential between an ordinary hydrogen electrode in the phosphate mixture and a hydrogen electrode in the same phosphate mixture saturated with quinol. But hydrogen electrodes turned out to give no constant and reproducible values in phosphate-quinol mixtures. Two electrodes mounted in the same electrode vessel differed by 5–6 millivolts, and besides the electrodes were considerably polarised during the readings. On the other hand, stable and reproducible potentials were obtained by means of the electrode recently described by Billmann and Klit [1927]. In this electrode colloidal palladium catalyses the reversible transformation hydrogen-hydrogen ion, and the electrodes are not platinised but simply bright platinum foils. The different ways in which the two electrodes comport themselves may be explained by the different efficiencies of the two electrodes, as in the electrodes the feeble traces of quinone (or quinhydrone) always present in quinol have to be reduced to quinol down to a quinone concentration which is quite below analytical limits, before stable potentials will be obtained. In a hydrogen electrode containing quinol we have also a quinolquinone electrode, and these two electrodes will only produce the same potential, when, by the reduction of the traces of quinone, equilibrium corresponding to the following reversible reaction is obtained.



At a hydrogen pressure of 1 atmosphere in the state of equilibrium the ratio conc. quinol/conc. quinone should be about  $10^{24}$ .

The stable and well-defined potential of the palladium hydrogen electrode in the phosphate quinol mixture was about 0.03 volt more positive than the unstable potential of the ordinary hydrogen electrode in the same mixture.

Tables VI and VII show the potentials between hydroquinhydrone electrodes in phosphate and borate buffers and the palladium hydrogen electrodes in the same buffers saturated with quinol.

Table VI.

+ Quinol, quinhydrone, phosph. mixt.   phosph. mixt. sat. quinol, Pd, $\text{H}_2$ - . 18°.							
$p_H$ of buff. mixt.	5.90	6.45	6.81	7.16	7.38	7.73	8.04
$\pi$	0.6194	0.6190	0.6189	0.6195	0.6193	0.6189	0.6194
Mean value: 0.6192.							

Table VII.

+ Quinol, quinone, borate mixt.   borate mixt. sat. quinol, Pd, $\text{H}_2$ - . 18°.			
$p_H$ of borate mixt.	7.34	7.76	8.05
$\pi$	0.6213	0.6201	0.6193

The figures show that in borate mixtures no mean value of  $\pi$  can be obtained, while in the phosphate mixtures the values of  $\pi$  are found about a mean value 0.6192. For the potentials of hydroquinhydrone electrodes to

hydrogen electrodes in solutions of hydrochloric or sulphuric acid Biilmann and Lund [1923] have found values from 0.6176 to 0.6181.

The depression  $\Delta p_H$  of the  $p_H$  value of the buffers produced by the saturation of the buffer with quinol may be determined by measuring the potential  $\Delta\pi$  between the palladium-hydrogen electrode in a buffer mixture saturated with quinol and the ordinary hydrogen electrode in the same buffer mixture *without* quinol. Table VIII shows the values of  $\Delta\pi$  and  $\Delta p_H$  for the buffer mixtures with which we are dealing, the original  $p_H$  of these being recorded in the first column of the table. In order to show variations, the  $p_H$  values (determined carefully with hydrogen electrodes) are given to 3 decimals; further, the total quinol concentration is recorded, the quinol being determined by titration with iodine in bicarbonate alkaline solution.

According to the definition of  $p_H$  the true  $p_H$  value of the buffer mixtures saturated with quinol is  $(p_H - \Delta p_H)$ . From the true  $p_H$  values, the quinol concentrations and the first dissociation constant of quinol it is possible to calculate with fair accuracy the influence which the quinol as an acid has on the  $p_H$  value of the mixtures.  $\Delta'p_H$  in Table VIII gives the figures. The values of  $\Delta''p_H = \Delta p_H - \Delta'p_H$  show that influences other than that of the acidity of quinol affect the determinations in the phosphate mixtures and that the matter seems rather complicated, while in the borate mixtures  $\Delta'p_H$  agrees fairly well with  $\Delta p_H$ .

Table VIII.

$p_H$	$\Delta\pi$	$\Delta p_H$	$\Delta'p_H$	$\Delta''p_H$	Quinol, molarity
<i>Phosphate mixtures.</i>					
5.896	0.0030	0.052	0.003	0.049	0.479
6.452	0.0035	0.061	0.006	0.055	0.472
6.811	0.0034	0.059	0.011	0.048	0.501
7.158	0.0040	0.069	0.031	0.038	0.526
7.382	0.0054	0.094	0.078	0.016	0.534
7.725	0.0108	0.187	0.177	0.010	0.540
8.040	0.0215	0.372	0.390	-0.007	0.555
<i>Borate mixtures.</i>					
7.338	0.0053	0.092	0.095	-0.003	0.480
7.760	0.0077	0.133	0.132	0.001	0.493
8.054	0.0104	0.180	0.183	-0.003	0.507

The measurements show that the hydroquinhydrone electrode is not as a rule applicable for determinations of  $p_H$  in phosphate and borate mixtures. On the other hand, in certain intervals of  $p_H$ , it may be applied, when the right constants or suitable comparison electrodes are employed. So it may be possible to use the hydroquinhydrone electrode when measuring against another hydroquinhydrone electrode in a phosphate mixture not differing essentially from the liquid examined, a method made use of by Biilmann and Katagiri [1927].

## V. COMPARISON OF THE DIFFERENT ELECTRODES.

Table IX shows determinations of  $p_H$  in the same mixtures with quinhydrone, quinoquinhydrone and hydroquinhydrone electrodes as well as with ordinary hydrogen electrodes and with the colloidal palladium hydrogen electrodes. The electrodes were measured against the quinhydrone standard electrode with  $p_H$  2.029 described by Veibel [1923].

According to the potentials given in sections II-IV the  $p_H$  values at 18° are calculated by means of the formula

$$p_H = 2.029 + \frac{\pi + \pi'}{0.05774},$$

in which  $\pi$  is the measured potential and  $\pi'$  has the following values according to the electrode type employed.

Hydrogen or coll. Pd, hydrogen electrode	$\pi' =$	-0.7042
Quinhydrone: in phosph. mixt.	$\pi' = 0.7058 - 0.7042 =$	0.0016
in borate mixt.	$\pi' = 0.7045 - 0.7042 =$	0.0003
Quinoquinhydrone	$\pi' = 0.7561 - 0.7042 =$	0.0519
Hydroquinhydrone	$\pi' = 0.8192 - 0.7042 =$	-0.0850

The table shows the figures from two series of measurements I and II, quite independent of each other and made with two preparations of the different buffer mixtures.

Table IX.  $p_H$  determined with different electrodes.

Hydrogen electrode		Quinhydrone electrode		Quinoquinhydrone electrode		Hydroquinhydrone electrode		Coll. Pd, H <sub>2</sub> sat. quinol	
I	II	I	II	I	II	I	II	I	II
<i>Phosphate mixtures.</i>									
5.90	—	5.89	—	5.90	—	5.83	—	5.85	—
6.45	6.46	6.46	6.47	6.46	—	6.40	6.41	6.39	6.41
6.81	6.81	6.81	6.81	6.81	6.82	6.75	6.76	6.75	6.76
7.16	7.17	7.16	7.17	7.16	7.18	7.08	7.09	7.09	7.09
7.38	7.39	7.39	7.39	7.38	(7.44)	7.28	7.30	7.29	7.31
7.73	7.73	7.72	7.73	—	—	7.53	7.55	7.54	7.53
8.04	8.04	8.02	8.01	—	—	7.66	7.69	7.67	7.67
<i>Borate mixtures.</i>									
7.34	7.34	7.33	7.34	—	—	—	—	7.25	7.25
7.76	7.75	7.75	7.75	—	—	—	—	7.63	7.61
8.05	8.06	8.05	8.07	—	—	—	—	7.87	7.88

The agreement between the hydrogen electrode and the quinhydrone electrode as well as the quinoquinhydrone electrode is excellent in the phosphate mixtures examined, and the quinhydrone electrode gives exact values in the borate mixtures.

The  $p_H$  values found with the hydroquinhydrone electrode in phosphate mixtures are not to be compared with those found with the hydrogen electrode, but with the values found with the colloidal palladium hydrogen electrode, and it will be seen that the figures agree excellently. *Of course none of these electrodes gives the  $p_H$  as it was before the saturation with quinol, but the  $p_H$  of the mixture after saturation with quinol.*

As to the borate mixtures, no determinations of  $p_H$  could be made with quinoquinhydrone or hydroquinhydrone electrodes, because no mean values of the potentials between these electrodes and the hydrogen electrode (see section IV) and the colloidal palladium hydrogen electrode respectively (see Table V) could be determined.

# VI. REMARKS ON BIILMANN AND KATAGIRI'S PAPER [1927] AND ON F. GROSSMAN'S PAPER [1927].

Biilmann and Katagiri have applied the hydroquinhydrone electrode to some determinations of  $p_H$  in liquids containing glucose. They made use of a phosphate mixture, the  $p_H$  of which was found to be 6.79 by means of the quinhydrone electrode. According to the reduction potential of the quinhydrone in phosphate mixtures determined in section II, the true  $p_H$  value of the said solution is calculated to be 6.81, which agrees completely with the value determined with the hydrogen electrode (see Table IX).

In the use of the hydroquinhydrone electrode Biilmann and Katagiri have not introduced any correction for the acid effect of quinol, and this is quite allowable, as the  $p_H$  values of the mixtures are 6.81 or less, and, at 6.81, the correction would only amount to 0.01. On the other hand, the influence of quinol on the activities of the ions stated in the present paper has to be considered. In many of the measurements this influence is automatically eliminated, as Biilmann and Katagiri employed a hydroquinhydrone electrode containing an electrolyte of almost the same kind as the liquid examined. But as far as solutions saturated with  $CO_2$  are concerned, the influence of quinol on such solutions cannot be judged from the measurements recorded above in the present paper.

In order to find out the  $p_H$  values of phosphate mixtures saturated with  $CO_2$  we have calculated the value by means of the constants of carbonic and phosphoric acids as well as determined the  $p_H$  value with the ordinary quinhydrone electrode.

Table X.

$p_H$ of $Na_2HPO_4$ solutions saturated with $CO_2$ at 735 mm. Hg. 18°.			
$Na_2HPO_4$	0.025	0.050	0.100 M
Biilmann and Katagiri	6.05	6.26	6.45
Calculated	6.02	6.22	6.42
Quinhydrone	6.01	6.21	6.39
Biilmann and Katagiri corr.	6.01	6.22	6.41

Table X deals with the solutions treated in Table VIII of the paper by Biilmann and Katagiri. The values from the cited Table VIII are shown in the first row. The second row shows the calculated values and the third the values determined by means of the simple quinhydrone electrode. The figures show that the  $p_H$  values found by Biilmann and Katagiri are about 0.04 too high. The origin of this difference is that Biilmann and Katagiri, in their paper, assume the  $p_H$  of the phosphate hydroquinhydrone electrode to be 6.79 at 18°, but its true value is 6.75 as shown above. The hydroquinhydrone

having no, or only a slight, influence on the  $p_H$  of the phosphate mixture saturated with  $\text{CO}_2$ , the true  $p_H$  value of the comparison electrode ought to be used. The fourth row shows the  $p_H$  values from the measurements of Büllmann and Katagiri corrected in this manner.

Table XI.

$p_H$ of $\text{NaHCO}_3$ solutions saturated with $\text{CO}_2$ at 740 mm. Hg. 25°.			
$\text{NaHCO}_3$	0.025	0.050	0.100 <i>M</i>
Büllmann and Katagiri	6.22	6.45	6.74
Calculated	6.13	6.40	6.66
Quinhydrone	6.11	6.37	6.64
Büllmann and Katagiri corr.	6.15	6.38	6.67

Table XI deals, in the same manner, with Büllmann and Katagiri's experiments with bicarbonate solutions saturated with  $\text{CO}_2$  at 25° (Table XII of the paper quoted). The table shows that the true  $p_H$  values in this case are about 0.07 lower than those found by Büllmann and Katagiri. This agrees with the fact that the  $p_H$  of the phosphate mixture of the comparison electrode saturated with quinol at 25° is not 6.79 but 6.72 as now found by means of the colloidal palladium electrode. The accordance between the figures in Table XI is not as good as in Table X, but suffices to show the order of magnitude of the correction which has to be introduced.

According to the facts mentioned in this section the electrometrically determined  $p_H$  values of solutions saturated with  $\text{CO}_2$  in Büllmann and Katagiri's paper ought to be corrected. The true  $p_H$  values of the solutions saturated with  $\text{CO}_2$  at 18° are 0.04, and those saturated with  $\text{CO}_2$  at 25° are 0.07 lower than indicated, while the differences between the  $p_H$  values in each series of measurements are not affected by the correction.

Finally we shall make some remarks concerning the paper of Grossman [1927] on the use of the hydroquinhydrone electrode for  $p_H$  determinations in the fluids of the organism. Grossman has observed that the directly measured  $p_H$  values between 7 and 8 need a correction  $\Delta p_H$ , and for this correction he gives the formula

$$\Delta p_H = 0.24 \times 2^{p_H - 7} - 0.21,$$

where  $p_H$  in the exponent is the directly found  $p_H$  value. It seems to us that a formula of this type will only hold good for a certain buffer in a certain concentration, as the acid influence of the quinol may depend on the buffer effect of the examined mixture. A direct comparison with our measurements is difficult, as Grossman has carried out his experiments at different temperatures and the potentials of the hydroquinhydrone electrodes depend on the temperature. So we found for the chain

$\text{H}_2$  - phosphate mixture  $p_H$  6.81 - hydroquinhydrone

the potential 0.6229 volt at 18°, and 0.6189 volt at 25°. According to this the potential of the chain at a temperature  $t$  between 18° and 25° is

$$\pi = 0.6229 - 0.00057 (t - 18) \text{ volt.}$$

For the corresponding chain containing 2 *N* sulphuric acid Schreiner [1925] has found the constant 0.000651 between 12° and 22° and 0.000641 between 22° and 32°. These values agree so well with the factor 0.00057 that we have calculated the potentials at 18° for the phosphate mixtures examined by Grossman at 19°. Further, from our values in Table IV we have calculated by means of graphic interpolation the potentials of mixtures which have not been directly measured by us. The values are recorded in Table XII. The values for the mixtures Nos. 1 and 3 are taken directly from Table IV, whereas the values for the mixtures Nos. 2 and 4 are found by interpolation.

Table XII.

No.	Phosphate mixture (cc.)		Grossman	Biilmann and Klit
	1/15 <i>M</i> $\text{Na}_2\text{HPO}_4$	1/15 <i>M</i> $\text{KH}_2\text{PO}_4$		
1	50	50	0.6165	0.6229
2	61	39	0.6203	0.6233
3	80	20	0.6241	0.6253
4	87	13	0.6259	0.6272

It will be seen that in most cases the two series of potentials do not agree so well as might be desired for the  $p_{\text{H}}$  determinations. Further, we shall point out that the way in which borate mixtures act with quinol is so different from the manner in which the phosphate mixtures are affected by quinol that we do not consider that a common formula will hold good for the corrections in both cases.

## SUMMARY.

1. The simple quinhydrone electrode and the quinoquinhydrone and the hydroquinhydrone electrodes have been examined in phosphate and borate mixtures by comparison with hydrogen electrodes in the same mixtures and the potentials of chains of these types are determined for phosphate mixtures with  $p_{\text{H}}$  5.90–8.04 and borate mixtures with  $p_{\text{H}}$  7.34–8.05.

2. The simple quinhydrone electrode gave correct  $p_{\text{H}}$  values in phosphate mixtures up to 7.73 and still very good values at  $p_{\text{H}}$  8.04; in the borate mixtures it gave correct values.

3. The quinoquinhydrone electrode gave correct values in phosphate mixtures up to  $p_{\text{H}}$  7.38. In more alkaline phosphate mixtures and in the borate mixtures the potentials were very drifting.

4. The hydroquinhydrone electrode gave very stable potentials in the phosphate mixtures, but too low  $p_{\text{H}}$  values because of the acid influence of the hydroquinhydrone. The influence of the quinol was determined by means of the colloidal palladium hydrogen electrode. The influence may be eliminated by using a comparison electrode of a similar type.

5. Owing to the acid influence of quinol, corrections must be introduced in the measurements of Biilmann and Katagiri with hydroquinhydrone electrodes in phosphate and bicarbonate solutions.



6. It is shown that the acid influence of quinol may affect the measurements of Grossman in phosphate and borate mixtures in such a manner that the correction formula of Grossman may only hold for special cases.

#### REFERENCES.

- Billmann (1921). *Ann. Chim.* (9), 15, 109.  
—— (1927). *Bull. Soc. Chim.* (4), 41, 213.  
Billmann and Katagiri (1927). *Biochem. J.* 21, 441.  
Billmann and Klit (1927). *Z. physikal. Chem.* 130, 566.  
Billmann and Lund (1923). *Ann. Chim.* (9), 16, 321.  
Grossman (1927). *Biochem. J.* 21, 267.  
Schreiner (1925). *Z. physikal. Chem.* 117, 57.  
Veibel (1923). *J. Chem. Soc.* 123, 2203.

## CVIII. THE PHOSPHATASES OF MAMMALIAN TISSUES.

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(Received April 27th, 1928.)

AMONGST the many phosphorus compounds which are to be found in almost every tissue of the animal body, in addition to inorganic phosphate, phosphatides and nucleoproteins, there are also present phosphoric esters of an acid-soluble nature, stable in dilute acid or alkaline solution and of relatively small molecular dimensions. The amount of phosphorus present in this ester form, in most of the tissues, is considerably in excess of that present as inorganic or phosphatide phosphorus. Enzymes capable, under certain conditions, of hydrolysing these important esters are also widely distributed in the body, and in certain tissues have been assigned specific functions which if confirmed would give these catalysts a position of very great metabolic importance.

The present paper deals with (a) the quantitative distribution of phosphatases in mammalian tissues, (b) the question as to whether a specific enzyme is required for the hydrolysis of each phosphoric ester, and (c) the synthetic activity of the phosphatases.

### A. THE QUANTITATIVE DISTRIBUTION OF PHOSPHATASES IN CERTAIN MAMMALIAN TISSUES.

Grosser and Husler [1912], Plimmer [1913, 1], Forrai [1923] and Robison [1923] have described experiments in which ground-up tissues or tissue extracts have been shown to have differing hydrolytic activity towards glycerophosphate or other phosphoric esters, but quantitatively these results can only be considered preliminary.

The determination of the quantitative distribution of any enzyme in the tissues is, for several reasons, a problem of quite a different order from that of the distribution of a particular element or compound. In the case of an enzyme, (1) it may be found impossible to remove the enzyme from the tissues without destroying its activity, (2) extracts of different tissues may contain in addition to the enzyme greater or lesser amounts of accelerating or inhibitory substances, (3) the activity of the enzyme will vary with the  $c_H$ , giving an optimum which may be different for the different tissues, (4) the functional  $c_H$  for most intracellular enzymes is unknown, so that it is not possible to compare activities at such hydrogen ion concentrations, (5) the choice of substrate is important; the "natural" substrate may be unknown.

It is impossible to avoid all these potential sources of error; we can perform the determinations neither under strictly "natural" nor strictly artificial conditions, and a compromise is inevitable.

One fortunate preliminary finding for the phosphatase activity of all tissue extracts examined is that the optimum  $c_H$  is very nearly the same, whether the "natural" esters, separated from the tissues by precipitation of the proteins, or esters of known composition such as sodium glycerophosphate, sodium hexosediphosphate or the sodium salt of guaninenucleotide are used.

As enzyme preparations, tissue hashes have been tried; also the fluids obtained by extraction of the ground-up tissue with dilute acid and alkali, and with glycerol containing various amounts of water, of acid or of alkali. Extraction with very dilute acids, or with acid glycerol, gives poor yields. The method found to be most practicable, which gives enzyme activities on the whole greater than any other method, is simple extraction of the finely ground-up tissue with chloroform water for 24-48 hours at room temperature, followed by slow filtration through cotton wool. The turbid filtrate contains almost all of the activity of the original tissue. Extracts of gastric and intestinal mucosa are prepared as follows. In the case of the rabbit or the cat, the whole intestine is washed out well with 0.9 % NaCl, then, after draining, the first 8 inches of the small intestine are taken as duodenum, the rest of the intestine as far as the caecum is then divided into two halves, the upper half is called jejunum and the lower ileum. The mucosa is scraped off with the back of a scalpel, weighed and extracted as described. With regard to accelerating or inhibiting substances, it is found that a short dialysis (18 hours) against 20 volumes of water has little effect on the activity of either kidney or duodenal extracts, although 6 days' dialysis against repeated changes of water shows some inactivation, which in the case of kidney extracts can be partially restored by the addition of a boiled extract of kidney.

For purposes of determining the relative distribution of the enzyme in the tissues the extracts are not purified further. The method of determining the relative activity has been standardised as follows. A small portion of tissue extract (0.5 to 1 cc.), representing a known weight of original tissue and brought to the optimal  $c_H$  of the enzyme, is added to a number of tubes each containing excess of the substrate (5 cc.) in glycine-NaOH buffer (5 cc.) at a short series of  $c_H$  near to the optimum, and incubated at 37° in presence of chloroform (which is found to have a negligible effect on the activity) for exactly 2 hours. The hydrolysis is stopped by the addition of trichloroacetic acid (2 cc. of 25 %) and the amount of inorganic phosphate liberated determined by the Briggs method. Controls containing substrate + buffer, and enzyme + buffer are put up at the same time. The correction for autolysis is usually very small. The number of enzyme units per g. of original tissue is then calculated, a unit being defined as the amount of enzyme, at the optimal  $c_H$  for its activity, which liberates 1 mg. P under the above conditions. For sodium glycerophosphate, or sodium hexosephosphate or the "natural"

esters of the tissues this optimum is found to be at  $p_H$  8.8–9.1; for the sodium salt of guaninenucleotide it is usually  $p_H$  9.0–9.2.

In Table I are shown the average values for the tissues of the rabbit, the cat and man, with glycerophosphate as substrate. In Fig. 1 the relative activity of certain tissues of the cat towards three substrates—glycerophosphate, hexosediphosphate, nucleotide—is shown. Table II shows a comparison of two methods of extraction; the optimal  $c_H$  for phosphatase activity of extracts obtained by the two methods appears to be the same.

Table I. *Average phosphatase content of the tissues.*  
*Substrate, sodium glycerophosphate.*

Figures indicate average enzyme units per g. of original tissue (wet weight).

Tissue	Animal and number examined		
	Rabbit (3)	Cat (3)	Man (2)
Gastric mucosa ...	0.2	1.5	0.5
Duodenal mucosa ...	15.2	27.5	7.7
Jejunal mucosa ...	30.7	29.7	11.5
Ileal mucosa ...	16.1	23.9	13.6
Colon mucosa ...	5.3	10.0	3.7
Liver ...	3.6	1.1	0.8
Lung ...	3.2	7.6	1.0
Kidney ...	10.5	14.1	4.8
Spleen ...	5.6	0.9	1.0
Pancreas ...	—	0.7	—
Parotid gland ...	—	1.7	—
Suprarenal gland ...	—	—	1.3
Brain ...	1.0	0.8	0.6
Cardiac muscle ...	0.3	0.2	—
Skeletal muscle ...	0.2	0.2	—
Bone ...	6.0	3.0	—
Artery ...	—	Nil	0.1

Table II. *Two methods of extraction compared.*

Units phosphatase per g. of tissue.

Tissue	Rabbit Tissues extracted with		Man Tissues extracted with	
	Chloroform water	60 % glycerol	Chloroform water	60 % glycerol
Gastric mucosa ...	0.2	0.2	0.6	0.5
Duodenal mucosa ...	8.7	8.7	12.0	7.0
Jejunal mucosa ...	21.0	13.5	13.3	Lost
Ileal mucosa ...	11.8	12.2	19.9	16.5
Colon mucosa ...	5.6	6.4	—	—
Liver ...	3.8	3.7	0.7	0.7
Lung ...	2.5	4.6	0.8	0.6
Kidney ...	12.6	11.8	3.4	3.3
Spleen ...	4.5	5.1	1.3	0.8
Brain ...	1.4	1.5	0.3	0.4
Heart ...	0.3	0.3	0.2	0.2
Muscle ...	0.2	0.2	—	—

There is a surprising similarity between the distribution in animal tissues of glycerophosphatase (or hexosephosphatase or nucleotidase) and that of ereptase, the enzyme investigated by Vernon [1904, 1905], which hydrolyses

peptones to substances which do not give the biuret test. Two examples of this similarity are given in Tables III and IV<sup>1</sup>.

Table III. *Enzyme distribution in cat's tissues.*

Tissue	Enzyme		
	Glycerophosphatase Mean of 3	Hexosediphosphatase One individual	Ereptase (Vernon) Mean of 2
Gastric mucosa ...	1.5	2.2	3.9
Duodenal mucosa ...	27.5	53.3	27.7
Jejunal mucosa ...	29.5	45.7	18.2
Ileal mucosa ...	23.9	24.6	14.4
Colon mucosa ...	10.0	11.7	5.8
Liver ...	1.1	1.6	5.0
Lung ...	7.6	[3.8]*	6.9
Kidney ...	14.1	22.9	14.3
Spleen ...	0.9	2.2	7.6
Pancreas ...	0.7	—	6.4
Brain ...	0.8	[1.0]*	1.2
Cardiac muscle ...	0.2	1.5	1.6
Skeletal muscle ...	<0.2	0.4	0.8

\* From a second cat.

Table IV. *Phosphatase and ereptase in the kidneys of various animals.*

	Units (average)			
	Rabbit	Cat	Man	Guinea-pig
Ereptase (Vernon)	[8] 10.9	[8] 11.6	[2] 5.2	[7] 8.8
Glycerophosphatase	[5] 11.4	[3] 14.1	[11] 4.8	[1] 7.9

Figures in brackets show number of individuals.

Phosphatase and ereptase also appear to be extractable from the tissues in the same way, to have their optimal activity in the same region of hydrogen ion concentration, and to have similar stabilities. In addition there are also remarkable correspondences (a) between the amount of ereptase and phosphatase in various organs at different stages in foetal and post-natal life (compare Vernon [1905] and figure given by Kay [1926, 1]) and (b) between the amounts in healthy and in diseased organs (Table V).

Table V. *Enzyme activity in diseased and healthy kidneys.*

	Phosphatase units [Brain and Kay, 1927] Average	Ereptase units [Vernon, 1908] Average
Healthy kidneys	[11] 4.8	5.4
Parenchymatous degeneration	[7] 3.7	3.4
Advanced nephritis	[9] 1.6 to 0.4	2.8 to 0.86

Figures in brackets show number of individuals.

The significance of this curious correlation between the enzyme hydrolysing phosphoric esters and the enzyme hydrolysing peptones is difficult at present to evaluate, but points to some functional relationship in the cell between these apparently unconnected activities.

<sup>1</sup> By accident the numerical values of the units by which the activities of the two enzymes glycerophosphatase and ereptase are expressed are almost the same.

## B. POSSIBLE IDENTITY OF CERTAIN PHOSPHORIC ESTERASES.

Extracts of various tissues, prepared as just described, will hydrolyse a number of naturally occurring, and of synthetic phosphoric esters. Is the agent responsible for these hydrolyses the same, or is there a specific phosphatase for each substrate?

There is a good deal of cumulative evidence in favour of the identity of the glycerophosphatase with the hexosediphosphatase (at least in the liberation of the first half of the phosphoric acid) and with the nucleotidase of mammalian tissues. This evidence is as follows.

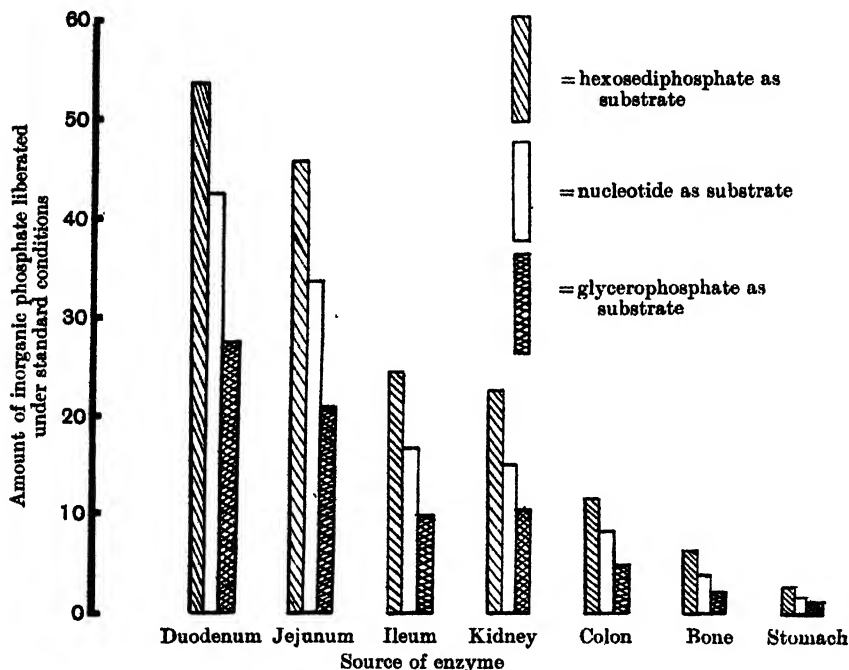


Fig. 1. Possible identity of glycerophosphatase, hexosediphosphatase and nucleotidase.

(a) *Optimal  $c_H$  for hydrolysis.* If a tissue preparation attacks different substrates at a different optimum  $c_H$ , it does not necessarily follow that different enzymes are concerned. But if a tissue preparation attacks closely related substrates at the same optimum  $c_H$ , there is a strong likelihood that the same enzyme is responsible. It has already been shown [Kay, 1926. 2]. that the hydrolysis of both sodium hexosediphosphate and sodium glycerophosphate by kidney phosphatase has the same optimum  $c_H$ . The same optimum  $c_H$  has since been found for the action of the various intestinal extracts and of lung, liver and bone phosphatase on these two substrates, though usually the optimum  $c_H$  for glycerophosphate hydrolysis by these

extracts is at about  $p_H$  9.0, and for hexosephosphate at about  $p_H$  9.1. With the same extracts guaninenucleotide (pure specimen prepared by the method of Buell and Perkins [1927] from yeast nucleic acid) is hydrolysed at an optimum  $p_H$  of about 9.2. These optima are thus sufficiently near to one another to arouse the suspicion that the enzymes concerned are not very dissimilar.

(b) Using extracts from various tissues to hydrolyse the same three substrates under the same conditions (of organic phosphorus concentration per cc., time, temperature, quantity of enzyme) it is found that there is a constant ratio between the amounts of inorganic phosphate liberated from the three substrates by any one of these extracts. This is sufficiently well indicated by Fig. 1 to require few further remarks. A similar ratio holds for human and rabbit tissues as for those of the cat (from which the values shown in Fig. 1 are derived), and this same ratio holds good within a small experimental error whether the tissue extracts are made with chloroform water or with 60 % glycerol.

(c) If two of the above substrates in the same solution are together exposed to the action of *small quantities* of such tissue extracts, the total liberation of inorganic phosphate is considerably less than what would be expected if two independent enzyme systems were present. It is, of course, essential that only small quantities of the hydrolysing agent be used, in order to keep the enzyme working at maximal activity during the period of the experiment. In Table VI are given the results of a few such experiments.

Table VI. *Identity of glycerophosphatase, hexosediphosphatase and nucleotidase.*

P in mg. liberated in 2 hours at 37.5°.

Substrate used*	Enzyme used, extract of			
	Cat's duodenum	Rat's kidney	Rat's bone	Cat's duodenum
Glycerophosphate	0.064	0.058	0.169	0.304
Hexosediphosphate	0.136	0.120	0.246	—
Nucleotide	—	—	—	0.299
Two together:				
(a) found	0.132	0.089	0.180	0.301
(b) calculated if two independent enzymes present	0.200	0.178	0.415	0.603

\* The organic P concentration of the substrates is not necessarily the same.

(d) If sodium fluoride is added to the reaction mixture containing a phosphatase and either glycerophosphate or hexosediphosphate, there is less hydrolysis during a given time than in the absence of this salt. Sodium glycerophosphate and sodium hexosediphosphate of the same original concentration of organic phosphorus have been hydrolysed by the same tissue extract in presence of increasing amounts of sodium fluoride, and it has been found that the percentage inhibition runs approximately parallel in both cases. With both substrates the effect of sodium fluoride may be just detected in as

low a concentration as  $M/100$ , the inhibition is 25–35 % in  $M/25$  and 60–70 % in  $M/6$  fluoride. The fact that an inhibiting agent interferes with the hydrolysis of both phosphoric esters at about the same rate is another small piece of evidence in favour of the identity of the two enzymes concerned. Sodium fluoride also inhibits the hydrolysis of nucleotide, but comparative quantitative experiments have not been made.

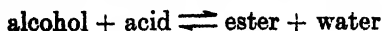
György [1925] has shown that phosphatolysis in all the tissues he examined (blood, liver, kidney, brain, heart muscle) appears to have a similar mechanism. It is stimulated by hydroxyl, by a low concentration of lactate or by potassium ions, checked by hydrogen, calcium and (a high concentration of) lactate ions.

It is submitted that although each of the foregoing points is, by itself, far from sufficient proof of the identity of the three enzymes, yet taken together they make a strong case in favour of such identity.

### C. SYNTHETIC ACTIVITY OF THE PHOSPHATASES.

To the short list of enzymes proved to be capable of synthetic activity must be added tissue phosphatase. Previous workers (Bodnár [1925], Martland and Robison [1927]) have shown that under certain conditions inorganic phosphate disappears in presence of tissue extracts derived either from the plant or the animal. Embden and Zimmermann [1924] have isolated hexosediphosphoric acid from the reaction mixture after allowing muscle press juice to act at low temperature ( $10^{\circ}$ ) on glycogen plus inorganic phosphate in presence of sodium fluoride. The reaction is evidently a complex one. So far this is the only case on record in which a phosphoric ester has been isolated following the synthetic enzymic activity of a tissue extract.

Using tissue extracts prepared as described earlier in the present paper, the enzymic synthesis of certain phosphoric esters can be accomplished with great ease at  $37^{\circ}$  and at  $p_H$  8–9, provided the alcohol concerned is present in sufficiently large excess in the reaction mixture. The balanced reaction



is thus pushed over toward the right hand side (the alcohol concentration being high and constant and the effective water concentration low). Synthesis goes on until an equilibrium is reached, the position of which depends in practice mainly on the amount of alcohol present.

Preliminary experiments have been carried out by mixing sodium phosphate solutions at  $p_H$  about 8.4 with duodenal or kidney extracts, and adding various alcohols or strong aqueous solutions of alcohols to the mixture. A sample of the reaction mixture is then taken at once, the proteins precipitated and inorganic and total phosphorus determined in an aliquot portion of the filtrate. The reaction mixture is raised to  $37.5^{\circ}$  and at intervals samples are taken and their content of inorganic and total phosphorus determined. (Controls using boiled enzyme are invariably used, and in no case show appreciable change in the concentration of inorganic phosphate.)



By this means the following facts have been established:

(a) Both kidney and intestinal extracts are capable of esterifying phosphoric acid in presence of high concentration of methyl or ethyl alcohol, ethylene glycol or glycerol.

(b) With propyl alcohol and dextrose, although definite synthesis has been observed, the amounts so synthesised have been very small.

(c) The products formed from glycerol (and glycol) were stable to 1 % sodium hydroxide at 100° for 4 hours at least. This is characteristic of glycerophosphates (Plimmer [1913, 2]).

(d) By simply diluting a portion of the reaction mixture with water, partial hydrolysis of the synthesised ester was usually observed.

(e) Sodium fluoride diminishes the *rate* of synthesis as of hydrolysis but probably does not affect the position of equilibrium. A typical experiment is shown in Table VII.

Table VII. *Effect of sodium fluoride on synthetic activity of phosphatase.*

Tube A. 1 cc.  $\text{Na}_2\text{HPO}_4$  solution at  $p_{\text{H}}$  8.4; 2 cc. cat's duodenal extract; 5 cc. glycerol, 2 cc. water.

Tube B. As A, but 1 cc. 0.8 M NaF (at  $p_{\text{H}}$  8.4) and 1 cc. water in place of 2 cc. water.

Tube C. As A, but 2 cc. 0.8 M NaF in place of 2 cc. water.

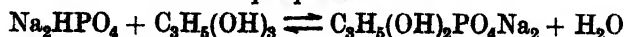
Tube D. As A, but enzyme first boiled.

Tubes E, F, G, H, as tubes A, B, C, D, but using kidney extract from same cat.

Time (days after start)	Percentage synthesis							
	Duodenal extract				Kidney extract			
	A	B	C	D	E	F	G	H
1	17	16	11	0	5	5	3	0
4	26	23	15	0	20	18	10	0
13	26	24	18	0	24	23	14	0
37	25	23	21	0	24	22	18	0

The above table also shows that the same position of equilibrium is reached with the extracts derived from two quite different tissues as catalysts.

The time relationships of the balanced reaction.  
phosphatase



have been studied. Two solutions are made up having the same amount of total P per cc.; in one, however, all the phosphorus is present as inorganic phosphate, in the other, all as sodium  $\beta$ -glycerophosphate, the  $p_{\text{H}}$  of each being 8.9. To each is added an equal volume of duodenal extract at  $p_{\text{H}}$  8.9 (previously dialysed for 18 hours) and twice the volume of neutral glycerol. Controls with inactivated enzyme are also made up.

The results are shown in Fig. 2, curves A, A. Curves B, B represent the results of a similar experiment in which the reaction has taken place in 75 % glycerol (by volume). In both experiments a further determination (not shown in the figure) has been made on the reaction mixtures after 6 days without showing significant change in the state of equilibrium.

From Fig. 2 it is clear that the same equilibrium position is reached whether inorganic sodium phosphate or sodium glycerophosphate is present to begin with, provided, of course, that water and glycerol are present in

amounts which are large relative to the amount of phosphorus (the concentration of P was only 22 mg. per 100 cc. of the reaction mixture). The position of the equilibrium, and the speed at which it is reached, are also seen to be dependent on the relative concentrations of glycerol and water.

Curves similar to those of Fig. 2 have also been obtained using kidney phosphatase as the catalyst.

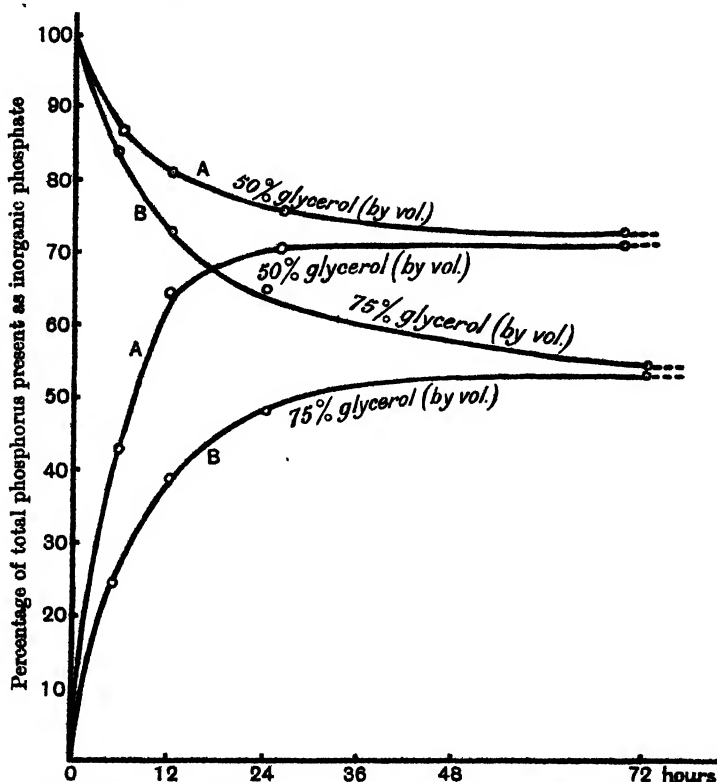


Fig. 2. Synthesis and hydrolysis of sodium glycerophosphate by duodenal phosphatase.

#### *Inhibitory effect of free phosphate.*

Free phosphate, even in relatively low concentrations is found to have a marked inhibitory effect on the hydrolysis of glycerophosphate by kidney or duodenal phosphatase (Table VIII).

Table VIII. *Inhibition of phosphatase by inorganic phosphate.*

Concentration of glycerophosphate at start	Concentration of inorganic phosphate at start	% inhibition after 2 hours' hydrolysis
M/40	Nil	0
"	M/400	4.6
"	M/200	12
"	M/133	30
"	M/100	62

Although low concentrations of phosphate thus markedly inhibit hydrolysis, free glycerol in much larger concentrations (up to about  $M/2$ ) has no such effect. Since the phosphatase is clearly specific, not for the alcohol portion, but for the phosphoric acid portion of the molecule (which is the only part common to all the esters) one would expect the phosphate configuration to be in close relationship with the structure of the enzyme molecule, and the finding that the activity of the enzyme is very sensitive to the presence of inorganic phosphate is not surprising.

*Isolation of products of synthesis by phosphatase.*

Sodium glycerophosphate has been isolated from the reaction fluid after allowing duodenal extracts to act on a mixture of sodium phosphate and glycerol for a week.

*Method.* 10 g. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  were dissolved in 100 cc. of extract of cat's duodenal mucosa (1 in 20) which had stood at room temperature in presence of chloroform for a week, and contained only traces of organic phosphorus. To this solution were added 160 cc. of pure glycerol. A sample of the liquid was taken at once for free and total P determination, and further samples at intervals afterward.

Time (days)	Free P (mg. per cc. liquid)	Total P	Organic P
0	3.60	3.61	0.01
4	2.91	—	0.70
7	2.39	—	1.22
10	2.36	3.66	1.30

After 10 days the reaction mixture was heated to  $100^\circ$  for 10 minutes to inactivate the enzyme, then diluted with an equal volume of water, and  $\text{Ca}(\text{NO}_3)_2$  added in slight excess followed by ammonia till no more precipitate was obtained. The mixture stood for a short time and was then filtered. The filtrate (500 cc.) contained no free phosphate, but 200 mg. organic phosphorus. It was boiled to remove ammonia, neutralised to litmus, and excess of lead acetate (25 %) added. After standing overnight the mixture was centrifuged. The supernatant liquid now contained no free phosphorus, but 14 mg. of organic phosphorus. After washing the precipitate twice with water it was resuspended in warm water and the lead removed by  $\text{H}_2\text{S}$ . The filtrate from lead sulphide now contained 6 mg. free P and 180 mg. organic P. 5 g. barium acetate were dissolved in it, and baryta was added until the reaction was alkaline to phenolphthalein. After filtering off the barium phosphate, barium glycerophosphate was precipitated by adding 4 volumes of alcohol. This process was repeated twice. The resulting product was dried *in vacuo* at  $100^\circ$  over phosphorus pentoxide. It weighed 1.60 g.

Found:	Ba, 44.5 %	P, 9.9 %
	44.6 %	
Calculated for $\text{C}_3\text{H}_7\text{O}_6\text{PBa}$	Ba, 44.7 %	P, 10.1 %.

A portion dissolved in water was precipitated by the exact quantity of sodium sulphate, and the filtrate found to contain organic phosphorus readily hydrolysed by kidney phosphatase but quite stable to 1 % NaOH at 100° for 6 hours. A portion of the barium salt gave the acrolein test for glycerol. The isolated salt was therefore the Ba salt of glycerophosphoric acid. Both the free acid and the barium salt in aqueous solution were optically inactive.

There is a distinction to be drawn between true phosphatases and other enzymes which are phosphatases incidentally. The true phosphatases, whose point of attack is the phosphoric acid portion of the ester molecule, are relatively stable enzymes. Tissue extracts kept at 0–4° in presence of chloroform retain their ability to hydrolyse phosphoric esters for a long time. One specimen of pig's kidney extract, which had an initial content of 0.31 unit per cc., had after 8 months a content of 0.26 unit, and other specimens have been kept for even longer periods without serious loss of activity. Dried tissues keep their phosphatase activity almost indefinitely. In muscle extracts prepared by the method described above there is very little of the true phosphatase. The activity towards glycerophosphate is practically nil, although there is distinctly more activity if hexosediphosphate is used as substrate. The difference is more marked with hashed muscle which hydrolyses hexosephosphate fairly readily but hardly attacks glycerophosphate. In the case of muscle extracts therefore the ratio between P liberated from hexosephosphate and from glycerophosphate by the same extract is anomalous and very different from the figure obtained under the same conditions using kidney, bone or intestinal extracts. Meyerhof [1926] found that an unstable enzyme could be separated from frog's or rabbit's muscle which hydrolysed glycogen and other polysaccharides to lactic acid, and also hydrolysed hexosephosphate, even after warming to 38°. Lactic acid being produced in the latter case also, the point of attack of this enzyme on the hexosephosphate molecule is clearly the alcohol radicle. It is probable that this explains the anomaly, the muscle extracts prepared by the ordinary extraction method still containing small quantities of the unstable *pseudo*-phosphatase. The true phosphatase appears not to produce lactic acid from naturally occurring phosphoric esters. The present author in conjunction with Dr J. T. Irving has recently found that kidney phosphatase will not produce lactic acid from the acid-soluble phosphoric esters of the kidney tissue, nor apparently from sodium hexosediphosphate<sup>1</sup>.

Meyerhof [1927] believes that the formation of a phosphoric ester is necessary before lactic acid can be formed from polysaccharides. Possibly one function of the true phosphatases *in vivo* is the synthesis of such esters. This possibility is supported by the experiments described in section C. There is no reason to believe that such synthetic activity would be limited to simple

<sup>1</sup> Duodenal phosphatase also hydrolyses fermentation hexosediphosphate without production of lactic acid. An extract of cat's duodenal mucosa hydrolysed 90 % of a solution of hexosediphosphate in 6 hours, producing 0.82 mg. inorganic P per cc., without producing any detectable quantity of lactic acid (i.e. less than 0.01 mg. per cc.).

esters. Since the phosphatases hydrolyse nucleotides (*in vitro*) with great ease, it is possible that under controlled conditions in the cell they are able to catalyse the synthesis of these phosphoric esters from nucleosides. This has not yet been attempted *in vitro*. Tissue phosphatases may similarly enter into the early stages in the formation of phosphatides. Further experiments on the synthetic activity of these enzymes are in progress.

It is nevertheless well established that *in vivo* the phosphatases may act hydrolytically. If sodium hexosephosphate or glycerophosphate be administered to an animal either intravenously or by mouth, the inorganic phosphate content of the blood and of the urine rises rapidly.

#### SUMMARY.

1. The phosphatases of mammalian tissues (*i.e.* the relatively stable enzymes present in extracts from such tissues which hydrolyse acid-soluble phosphoric esters) all appear to have an optimum  $p_H$  between 8.8 and 9.3. The character of the substrate influences this optimum to a slight extent. The distribution of phosphatase in the tissues of the rabbit, of the cat and of man has been quantitatively determined. This distribution is remarkably akin to that found for ereptase by Vernon. Other correspondences between these functionally widely dissimilar enzymes are described.

2. It is probable that the same enzyme is responsible for the hydrolysis of glycerophosphate, of hexosediphosphate and of nucleotide.

3. In presence of excess of the alcohol concerned, inorganic phosphate can be esterified, using phosphatases derived from various tissues as catalysts. Glycerophosphoric acid (isolated as the barium salt) has been synthesised in this way from glycerol, and several other alcohols have been shown to combine with inorganic phosphate. The equilibrium



has been studied, using phosphatase as catalyst.

The work has been carried out with the aid of a grant from the Research Fund of the London Hospital Medical College.

#### REFERENCES.

- Bodnár (1925). *Biochem. Z.* 165, 1.  
 Brain and Kay (1927). *Biochem. J.* 21, 1104.  
 Buell and Perkins (1927). *J. Biol. Chem.* 72, 21.  
 Embden and Zimmermann (1924). *Z. physiol. Chem.* 141, 225.  
 Forrai (1923). *Biochem. Z.* 142, 282.  
 Grosser and Husler (1912). *Biochem. Z.* 39, 1.  
 György (1925). *Biochem. Z.* 161, 157.  
 Kay (1926, 1). *Brit. J. Exp. Path.* 7, 177.  
 — (1926, 2). *Biochem. J.* 20, 791.  
 Martland and Robison (1927). *Biochem. J.* 21, 665.  
 Meyerhof (1926). *Naturwissenschaften*, 14, 196, 756.  
 — (1927). *J. Gen. Physiol.* 8, 631.  
 Plimmer (1913, 1). *Biochem. J.* 7, 43.  
 — (1913, 2). *Biochem. J.* 7, 72.  
 Robison (1923). *Biochem. J.* 17, 286.  
 Vernon (1904). *J. Physiol.* 32, 33.  
 — (1905). *J. Physiol.* 33, 81.  
 — (1908). *Intracellular enzymes*, 42. (John Murray, London.)

## CIX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

### I. THE RELATIONSHIP BETWEEN PHOSPHORIC ESTER ACCUMULATION AND PHOSPHORIC ESTER BREAK-DOWN AND LACTIC ACID FORMATION FROM GLYCOGEN.

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*(Received April 4th, 1928.)*

THE experiments recorded in this communication are part of an investigation into lactic acid and phosphoric ester formation from carbohydrates in muscle extract. The particular experiments recorded below deal with the supposed relationship of a quantitative nature between the lactic acid formation and the phosphoric ester accumulation and breakdown that accompany that process. Gottschalk [1926] gives a résumé of this subject and sets forth a series of reactions to explain the experimental results of Meyerhof. These results showed that in lactic acid formation from glycogen in muscle extract there are two distinct periods. In the first period there occurs a high rate of lactic acid formation and an accumulation of phosphoric ester. The extent of this accumulation is conditioned by the amount of carbohydrate or the amount of available phosphate. In the second period this accumulated ester is broken down and the rate of lactic acid formation diminishes. It is postulated that in the first period, for every two molecules of diphosphoric ester formed, one is immediately broken down to the end-product, lactic acid, while the other is "stabilised" and accumulates. This implies that for every two molecules of lactic acid formed, two molecules of inorganic phosphate are retained as ester, giving a molar ratio of lactic acid to phosphate retained as ester of unity. Since it is also put forward that the lactic acid formed in the second period originates from the ester accumulated in the first period, the molar ratio of lactic acid to increase in inorganic phosphate should again be unity.

It is to this aspect of the problem that the following experiments apply. Attention is drawn to the fact that the extracts employed here are, relatively speaking, fresh and concentrated. These extracts show different phenomena from those that have been kept for a short time at 37° prior to the addition of glycogen. This will be made the subject of a subsequent communication.

## EXPERIMENTAL PROCEDURE.

Cats were killed by a blow on the back of the head and were immediately perfused until free from blood, first with warm and then with cold Ringer's solution. The excised muscles were minced and then put through a grinding mill. The muscle pulp was ground up with silver sand in a mechanical mortar in lots of 350 g. A solution containing sodium bicarbonate and sodium chloride, both to the extent of 0.5 % and cooled to freezing point, was added in portions until about 350 cc. had been added in all. From the mortar the mixture was poured into a sheet of muslin and the extract was squeezed out in a hand-press. The combined extract was then frozen with stirring and filtered through a Büchner funnel without paper, thus separating the extract from the ice crystals. Repetition of this process soon reduces the volume to any desired extent. As a rule it took about 90 minutes to get the extract ready for incubation but every precaution was taken to keep the temperature low.

Before incubation more phosphate was added to the extent of 5 cc. of a 3 % solution of  $\text{NaH}_2\text{PO}_4$  per 100 cc. extract. The reaction was adjusted if necessary to about  $p_{\text{H}}$  7.2 by the addition of a saturated solution of sodium bicarbonate. In the experiments described below, the extract was divided up into lots of 160 cc. and placed in the incubation flasks. As 5 cc. were required for phosphate estimation and 10 cc. for the lactic acid estimation, one required for every withdrawal, at the appointed intervals, about 20 cc. The requisite volume of a freshly prepared 10 % solution of glycogen was measured out into very small flasks, water being added in the cases of the lower concentrations in order that the volume of fluid added might be the same in every case. The contents of the incubation flask were rapidly warmed to 37°, the glycogen solution was added and the mixture thoroughly shaken up. First, 5 cc. were pipetted out into 25 cc. of 2 % trichloroacetic acid and the mixture was at once frozen solid. It was kept in this condition until the phosphate analysis was done. Immediately after this 10 cc. were pipetted into a dry flask and 30 cc. of a tungstic acid solution added. (This tungstic acid was prepared just before the precipitation by mixing 10 cc. of a 5 % solution of sodium tungstate and 25 cc. of 0.5 *N* sulphuric acid.) The time was noted by an observer and the incubation flask immersed in a large water-bath at 37°. Each flask was fitted with a stopper holding three tubes. One reached to the bottom of the flask and through this was passed a stream of nitrogen, previously bubbled through a pyrogallol-potassium hydroxide solution. The second tube was a short elbow tube, not dipping into the extract, and served as a gas outlet. The third tube, like the first, dipped well under the extract; by closing the gas inlet by compressing the rubber connection and blowing gently through the gas outlet the extract could be siphoned out of the flask when it was desired to take a sample. If the second sample was to be taken 10 minutes after the start of the incubation only four incubation flasks could be used simultaneously with comfort, allowing for the time required for warming up the extract and for the pipetting. The

times of withdrawal were indicated by an assistant and were correct to a quarter of a minute.

The inorganic phosphorus was estimated by the method of Fiske and Subbarow [1925]. The colour was allowed to develop for 20 minutes in order to convert into inorganic phosphorus the whole of the labile phosphorus of phosphagen which was therefore included in the determinations of "inorganic phosphorus." The term "inorganic" phosphorus is thus really a misnomer, but as it was intended to investigate lactacidogen formation and breakdown, decrease and increase of "inorganic" phosphorus as estimated by the above method can be taken as indication of lactacidogen accumulation and breakdown. Under the above conditions lactacidogen and hexosediphosphoric acid are stable.

The lactic acid was estimated by the method of Clausen [1922], taking advantage of the improvements suggested by Shaffer [1927]. Interfering carbohydrates were removed by the usual copper-lime method. 20 cc. of the tungstic acid filtrate were accurately pipetted into a 50 cc. volumetric flask, 10 cc. of a 7 % lime suspension added, followed by 6 cc. of a 7 % copper sulphate solution. After shaking at intervals for half an hour, 2 cc. more of the lime suspension were added and the volume made up to 50 cc. This gives a final dilution of the extract of 1 in 10. 10 cc. of the filtrate of this were used for the oxidation by 0.005 *N*  $\text{KMnO}_4$ . The aldehyde was aerated into two tubes, the first holding 30 cc. of 0.5 *N* bisulphite and the second 20 cc. The excess of the unbound sulphite was titrated first with 0.1 *N* iodine and finally with 0.005 *N* iodine until just blue. Clausen underestimates the error incurred by overstepping this first end-point. The bound sulphite was titrated, with the gradual addition of saturated sodium bicarbonate solution, with accurate 0.005 *N* iodine. The results are based on a 95 % yield of acetaldehyde.

#### EXPERIMENTAL RESULTS.

The values for lactic acid and for "inorganic" phosphorus are expressed in the following tables as milligram-molecules or milligram-atoms respectively per 100 cc. extract multiplied by 100. Thus a concentration of 90 mg. lactic acid is represented by 100 as is also a concentration of "inorganic" phosphorus of 30.96 mg. per 100 cc. extract. A change of one unit in the tables indicates, for lactic acid, a change in concentration of 0.9 mg. and for phosphorus 0.3 mg. per 100 cc. extract. The figures can thus be used as molar quantities in any calculations.

*Exp. 237.*

	A. Glycogen 0.4 %		B. Glycogen 1.0 %	
	Extract 10 % glycogen Water	200 cc. 10 15	Extract 10 % glycogen	200 cc. 25
Time	Lactic acid	Phosphorus	Lactic acid	Phosphorus
Start	139	163	139	165
10 mins.	212	143	244	100
20 "	275	113	304	45
30 "	319	43	337	18
40 "	335	41	354	24
50 "	349	49	373	35
60 "	373	67	397	50
90 "	402	102	447	97



Exp. 240.	A. Glycogen 0.3 %			B. Glycogen 0.6 %			C. Glycogen 0.9 %		
	Extract	160 cc.		Extract	160 cc.		Extract	160 cc.	
	10 % glycogen	5		10 % glycogen	10		10 % glycogen	15	
	Water	19		Water	14		Water	9	
Time	Lactic acid		Phosphorus	Lactic acid		Phosphorus	Lactic acid		Phosphorus
Start	214		176	214		177	221		177
10 mins.	264		124	267		98	273		70
20 "	293		113	283		24	291		17
30 "	317		107	299		28	304		25
40 "	340		122	315		48	321		43
50 "	363		148	333		68	329		69
60 "	371		165	344		83	351		91
90 "	381		200	356		164	366		171

Exp. 241.	A. Glycogen 0.4 %			B. Glycogen 1.0 %		
	Extract	160 cc.		Extract	160 cc.	
	10 % glycogen	8		10 % glycogen	20	
	Water	24		Water	12	
Time	Lactic acid		Phosphorus	Lactic acid		Phosphorus
Start	222		180	220		190
10 mins.	289		122	309		86
20 "	330		150	370		93
30 "	373		152	421		116
40 "	412		156	464		132
50 "	452		169	518		145
60 "	473		191	577		149
90 "	480		218	707		154

*Period of ester accumulation.*

As the results show, the period of ester accumulation is a very variable quantity both in extent and duration. Exps. 237, 240 and 241 are considered in detail. The increase in lactic acid and the decrease in phosphorus are given for each interval examined and by dividing the former by the latter, the molar ratio of lactic acid formed to organic phosphorus accumulated as ester at the end of each period is obtained.

Exp. 237.	Glycogen 0.4 %			Glycogen 1.0 %					
	L.A.	Phos.	L.A.	L.A.	Phos.	Phos.	L.A.	Phos.	L.A.
Time interval (mins.)									
0-10	+73	-20	3.65	+105	-64	1.61			
10-20	+63	-30	2.10	+60	-55	1.09			
20-30	+44	-70	0.51	+33	-27	1.22			
0-30	+180	-120	1.50	+198	-147	1.35			
Exp. 240.	Glycogen 0.3 %			Glycogen 0.6 %			Glycogen 0.9 %		
	L.A.	Phos.	L.A.	L.A.	Phos.	Phos.	L.A.	Phos.	L.A.
0-10	+50	-52	0.96	+53	-79	0.68	+52	-107	0.49
10-20	+29	-11	2.63	+16	-74	0.22	+18	-53	0.34
0-20	+79	-63	1.25	+69	-93	0.74	+70	-160	0.44
Exp. 241.	Glycogen 0.4 %			Glycogen 1.0 %					
	L.A.	Phos.	L.A.	L.A.	Phos.	Phos.	L.A.	Phos.	L.A.
0-10	+67	-58	1.16	+89	-104	0.86			

These three experiments were made with extracts prepared in exactly the same manner from three different animals. The comparison between the low and the high concentrations of glycogen with the same extract indicates that

above a certain concentration of glycogen, ester accumulation is more powerfully influenced by the glycogen concentration than is the lactic acid formation. In Exp. 240 this is particularly so. In the first 10 minutes the lactic acid formation is practically the same for concentrations of 0.3, 0.6 and 0.9 % of glycogen, while, on the other hand, the ester accumulation with 0.9 % glycogen is double that with 0.3 %. With one exception, increasing the glycogen concentration leads to a decrease of the ratio of lactic acid to phosphorus accumulated as ester, by increasing the latter quantity out of proportion to the former.

*Period of breakdown of the accumulated ester.*

Analyses as above are now given for the second period with the same three experiments, the lactic acid formation and the phosphorus increase for every interval being compared.

Exp. 237. Time interval (mins.)	Glycogen 0.4 %			Glycogen 1.0 %			L.A.	Phos.	L.A. Phos.
	L.A.	Phos.	L.A. Phos.	L.A.	Phos.	L.A. Phos.			
40-50	+14	+ 8	1.75	+19	+11	1.73			
50-60	+24	+18	1.33	+24	+15	1.80			
60-90	+29	+35	0.83	+50	+47	1.06			
40-90	+67	+61	1.09	+93	+73	1.27			

Exp. 240.	Glycogen 0.3 %			Glycogen 0.6 %			Glycogen 0.9 %		
30-40	+23	+15	1.53	+16	+20	0.80	+17	+18	0.94
40-50	+23	+26	0.80	+18	+20	0.90	+ 8	+26	0.30
50-60	+ 8	+17	0.47	+11	+15	0.73	+22	+22	1.00
60-90	+10	+35	0.28	+12	+81	0.15	+15	+80	0.19
30-90	+64	+93	0.69	+57	+136	0.42	+62	+146	0.42

Exp. 241.	Glycogen 0.4 %			Glycogen 1.0 %		
10-20	+41	+28	1.48	+61	+ 7	8.71
20-30	+43	+ 2	21.5	+51	+23	2.21
30-40	+39	+ 4	9.9	+43	+16	2.69
40-50	+40	+13	3.07	+54	+13	4.15
50-60	+21	+22	0.95	+59	+ 4	14.7
60-90	+ 7	+27	0.26	+130	+ 5	26.0
10-90	+191	+96	2.0	+398	+68	5.85

In this period the above ratio is a very variable quantity. The onset of the breakdown of the ester accumulated in the first period is not in every case due to lack of glycogen but occurs in the presence of excess of glycogen. As a general rule, well demonstrated in Exp. 241, the value of the ratio diminishes as the glycogen concentration falls. When all the glycogen has been used up the ratio is invariably less than unity.

Two special cases are now given. The results of Exp. 239 are given in the accompanying graphs. Fig. 1 indicates the changes in "inorganic" phosphorus and Fig. 2 the changes in lactic acid both at intervals of 10 minutes, expressed in the same units as above. The peculiar feature here is the very slight accumulation of ester in the first 10 minutes with all three concentrations of glycogen but followed in the case of 1.0 % glycogen by a second

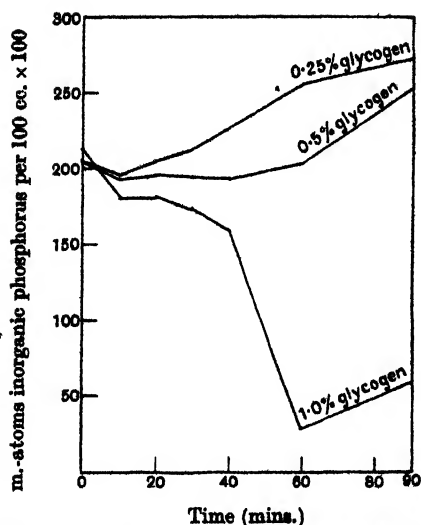


Fig. 1. Exp. 239. Phosphorus changes with glycogen concentrations of 0.25 %, 0.5 % and 1.0 %.

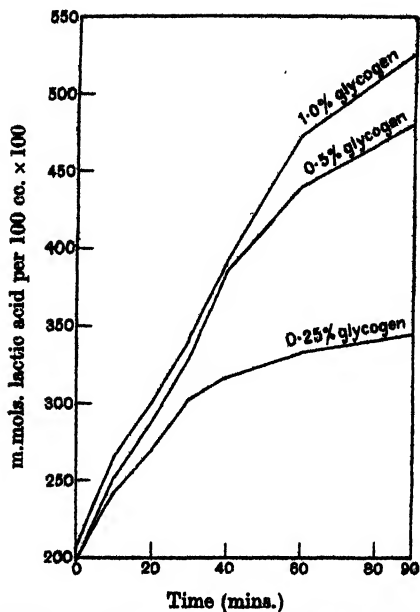


Fig. 2. Exp. 239. Lactic acid changes with glycogen concentrations of 0.25 %, 0.5 % and 1.0 %.

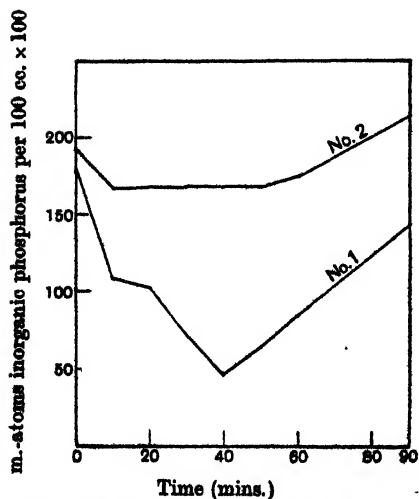


Fig. 3. Phosphorus changes of Exp. 243, No. 2 extract more concentrated than No. 1.

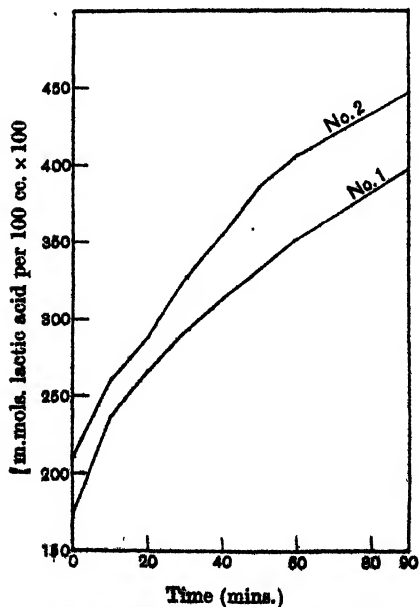


Fig. 4. Lactic acid changes of Exp. No. 243.

period of extensive ester accumulation. Contrasting 0.5 % and 1.0 % glycogen, it is shown that despite the great difference in behaviour of the phosphorus curves in the last 50 minutes, the lactic acid curves in these two cases are not very markedly different and the great ester accumulation with 1.0 % glycogen is not reflected in the corresponding lactic acid curve as it would be if these two processes were intimately related. Again, the difference between 0.25 % and 0.5 % glycogen is not so great in the phosphorus curves as it is in the lactic acid curves.

This same result was produced in Exp. 243 in the following manner. The muscles from two cats were mixed. Of the combined weight, 500 g. were extracted and gave 650 cc. extract, which was reduced by freezing to 450 cc. This is extract No. 1. The remaining 1200 g. muscle were extracted with a relatively smaller volume of extracting solution and yielded a litre of extract. This extract, No. 2, was not frozen at all. The  $p_H$  of both was adjusted to 7.3. The glycogen concentration in both cases was 0.3 %. The results are shown in Figs. 3 and 4. The outstanding feature here is the absence of ester accumulation in No. 2 extract. In No. 1 extract, on the other hand, ester accumulation is quite decided. On comparing the lactic acid curves it is seen that a high rate of lactic acid formation is not conditioned by, or related to, ester accumulation. This experiment demonstrates the danger of generalising from the results of extract No. 1, when by a slight alteration in the method of preparing the extract from the same batch of muscle, the results of No. 2 can be produced.

#### SUMMARY.

In the type of extract of cat muscle used in this investigation it has been found that phosphoric ester accumulation is not an essential accompaniment of lactic acid formation from glycogen. When ester accumulation does occur, there is no definite constant ratio of the molar amount of lactic acid produced to the molar amount of phosphorus that accumulates as ester. In the second period, when the ester that accumulates in the first period is broken down, the molar ratio of lactic acid produced to phosphorus set free is also very variable.

In concluding, the author would like to express his thanks to Prof. Waymouth Reid, F.R.S., for undertaking the perfusion of the animals.

#### REFERENCES.

- Clausen (1922). *J. Biol. Chem.* 52, 263.  
 Flake and Subbarow (1925). *J. Biol. Chem.* 66, 375.  
 Gottschalk (1926). *Ergeb. Physiol.* 25, 651.  
 Shaffer (1927). *J. Biol. Chem.* 73, 335.

## CX. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

### II. THE EFFECT OF SODIUM HEXOSEDIPHOSPHATE ON THE RATE OF ESTER ACCUMULATION DURING THE INCUBATION OF GLYCOGEN IN CERTAIN TYPES OF EXTRACTS.

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(Received April 4th, 1928.)

IN the first paper of this series it was stated that by a modification of the conditions in an extract, the balance between ester formation from glycogen and the co-existing breakdown of that ester could be changed. One method of producing this is by adding sodium hexosediphosphate along with the glycogen, and this method is described in detail in this paper.

The idea that prompted these experiments was the following. Sodium hexosediphosphate when incubated alone in muscle extract shows a steady liberation of inorganic phosphate and lactic acid, as Embden [1914] first showed. Glycogen, in the type of extract used here, shows the same steady rate of lactic acid formation, but in this case the accompanying phosphate changes are of three types, depending on the glycogen concentration and also on the nature of the extract. This classification is based on the results of investigations with about a hundred different extracts. These three types are indicated in Fig. 1: the graphs show the changes in inorganic phosphate plotted against the time of incubation.

Type 1. In this type there is no ester accumulation even with concentrations of glycogen as great as 0.8 %. The phosphorus concentration remains practically the same until all the glycogen has been used up, after which the phosphate increases. This increase is from pre-existing lactacidogen in the extract.

Type 2. In this type, no ester accumulation takes place during the first 30 to 40 minutes of the incubation, but thereafter an extensive and rapid accumulation takes place between 40 and 90 minutes from the start. This type is often produced by concentrations of glycogen of the order of 1.0 % in extracts that give a "type 1" curve with about 0.5 % glycogen.

Type 3. In this type the ester accumulation is intense at the very start, is completed in most cases in the first 10 to 20 minutes of the incubation period and is followed by a second period of breakdown of the ester.

The inorganic phosphate liberated in an extract of the type used here, to which no carbohydrate has been added, ranges from 25 to 35 mg. per 100 cc. This process is completed in about 60 minutes. This would indicate a certain concentration of lactacidogen because the liberation of the phosphate is always accompanied by lactic acid formation and the two processes slow down and stop together. It seemed not at all unlikely that the difference between a "type 3" and a "type 1" extract, for example, lay in the fact that the latter

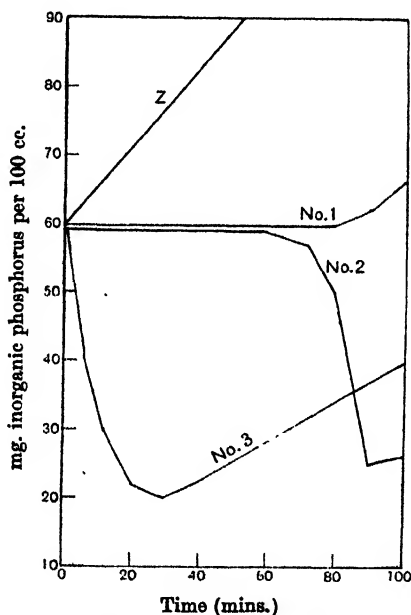


Fig. 1. Inorganic phosphorus changes.

Z. Plain sodium hexosediphosphate.  
 No. 1 type with glycogen.  
 No. 2 " "  
 No. 3 " "

type contained as a rule more lactacidogen than the former. The "phosphagen" content is likewise variable, but the present communication excludes investigations on the possible rôle of this labile phosphorus compound in this connection. This has been made the subject of investigation for a time in this laboratory and the results will be recorded in a subsequent communication. Considered here is solely the possible influence of the acid-stable lactacidogen and the related substance hexosediphosphate.

It appeared quite possible that if the lactacidogen were broken down at the same rate as the ester was being formed from the glycogen, the phos-

phorus liberated from the lactacidogen could be passed on to the glycogen (or to whatever breakdown product is converted into the ester) at a rate sufficient to keep the ester formation going at its maximum rate. This would spare, so to speak, the inorganic phosphate of the extract from taking part in this process, the concentration of inorganic phosphate would not change and the flat "type 1" curve would be produced. If the lactacidogen were not present to a great extent at the start, then the phosphorus liberated might not be set free at a rate great enough to keep the rate of ester formation at its maximum, and a call would then be made on the inorganic phosphate of the extract and thus a "type 3" curve might result. It was thought that, if, in the case of an extract yielding a "type 3" curve with glycogen, sodium hexosediphosphate were added along with the glycogen, the liberation of phosphate from this ester might be sufficiently rapid to balance the removal of phosphate by the esterification process, and thus a "type 3" curve might be converted into a flat "type 1" curve. The assumption made throughout is that lactacidogen or hexosediphosphate breakdown takes place in the presence of glycogen and simultaneously with the formation of ester from the latter. The phosphate curve is thus assumed to represent the balance between phosphoric ester breakdown and synthesis. As the experimental results will show, the production of a "type 1" curve is not conditioned by a high initial concentration of phosphoric ester; on the contrary the presence of a phosphoric ester along with glycogen either has no effect or leads to a decided accumulation of ester. Contrary to expectation a "type 1" curve is converted into a "type 3" curve.

#### EXPERIMENTAL PROCEDURE.

The extracts were prepared in exactly the same manner as those described in the first paper of this series, and when any special treatment is given it is stated. Two preparations of hexosediphosphate were used. One was prepared in this laboratory according to a private communication from Prof. Harden, using a dried yeast from the local brewery. The other preparation was a gift to the department from Bayer Products, Ltd. Both preparations yielded the same results.

The glycogen was added either as a 20 % or a 10 % suspension made up in boiling water. The sodium hexosediphosphate was added in the case of the Bayer product as a 20 % solution; in the case of the author's preparation, the strength of the stock solution, concentrated by distillation under reduced pressure, was determined from the difference between the inorganic phosphorus and the total phosphorus as estimated by the nitric acid method of wet ashing. In the case of the plain glycogen controls, water was added equivalent to the volume of the zymophosphate. The addition of these solutions and the method of sampling were as described in the previous paper.

## EXPERIMENTAL RESULTS.

In Exp. 217, a typical case, the phosphorus changes are represented in Fig. 2. All inorganic phosphorus concentrations are expressed as mg. per 100 cc. extract. Lactic acid estimations were not made in several of these preliminary experiments.

In Exp. 218 (Fig. 3) the presence of the zymophosphate shifted the period of maximum ester accumulation to an earlier point in the incubation. The higher concentration of zymophosphate is more effective than the lower.

In Exp. 219 the following were tried. Glycogen 0.33 % alone and with 1.2 % zymophosphate; glycogen 0.66 % alone and with 0.6 % and 1.2 % zymophosphate; glycogen 1.3 % alone and with these same two concentrations of zymophosphate. In those cases where the glycogen concentration

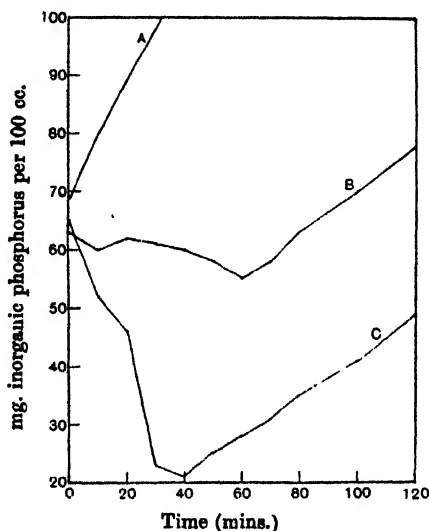


Fig. 2. Exp. 217.

- A. Zymophosphate 1.0 % alone.
- B. Glycogen 0.65 % alone.
- C. Glycogen 0.65 % with zymophosphate 1.0 %.

was 0.33 % or 0.66 %, none of the zymophosphate concentrations was effective. In the case of 0.33 % glycogen with 1.2 % zymophosphate, there was more phosphate liberated in the later stages of the incubation than with glycogen alone, indicating at this stage that, after all the glycogen had been broken down, the zymophosphate was hydrolysed. The results with the 1.3 % glycogen are shown in Fig. 4.

In Exp. 224, one-half of the extract was acidified to about  $p_H$  6.5 by HCl, the molar equivalent of NaCl being added to the other half which was adjusted to  $p_H$  7.5. The results are shown in Fig. 5. The acid extract gave the better result; the glycogen curve is a typical "type 1" curve and the effect of the zymophosphate is very decided.



In the next set of experiments the lactic acid formation was followed in addition to the changes in inorganic phosphorus. The lactic acid was estimated by the method mentioned in the preceding paper. The results of Exp. 238 are not given in detail. The phosphorus changes in a mixture of 1.5 % zymophosphate and 0.7 % glycogen were very similar to those in the 0.7 % glycogen alone. Nevertheless it was noticed that the total lactic acid formed with glycogen alone in 90 minutes was 167 mg. per 100 cc. extract and in the glycogen-zymophosphate mixture only 130 mg., a decrease of 22 %.

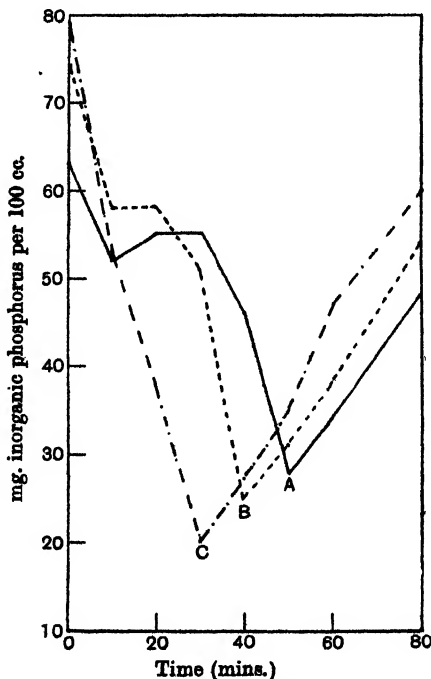


Fig. 3. Exp. 218.

- A. Glycogen 0.6 % alone.  
 B. " 0.6 % with zymophosphate 0.6 %.  
 C. " 0.6 % " 1.2 %.

In Exp. 240 glycogen 0.9 % alone and in conjunction with 1.0 % zymophosphate was investigated. There was a slight "extra ester accumulation" in this case associated with a 30 % decrease in the lactic acid production. The lactic acid produced in the plain glycogen was 130 mg. and in the glycogen plus zymophosphate mixture only 90 mg. on incubation for 90 minutes.

Exp. 241 was, however, a good case. Two concentrations of glycogen were tried with 1.0 % zymophosphate. In the case of 0.4 % glycogen, the zymophosphate did produce extra ester equivalent to 26 mg. of phosphorus. At the end of 60 minutes, the lactic acid increase in the plain 0.4 % glycogen was 226 mg. and in the corresponding glycogen-zymophosphate mixture only 167 mg., a decrease of 26 %.

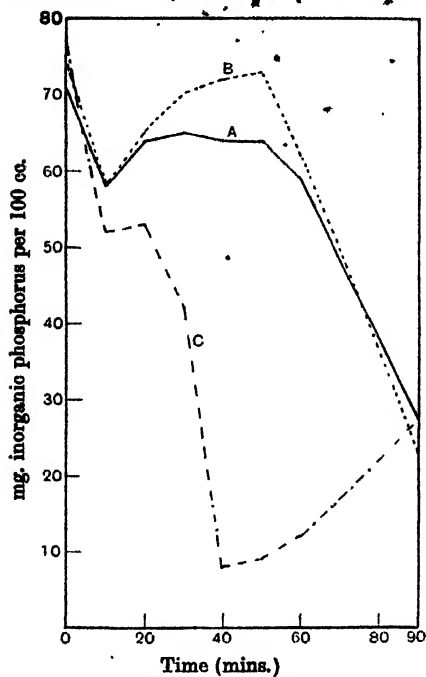


Fig. 4. Exp. 219.

- A. Glycogen alone 1.3 %.  
 B. " 1.3 % with zymophosphate 0.6 %.  
 C. " 1.3 % " 1.2 %.

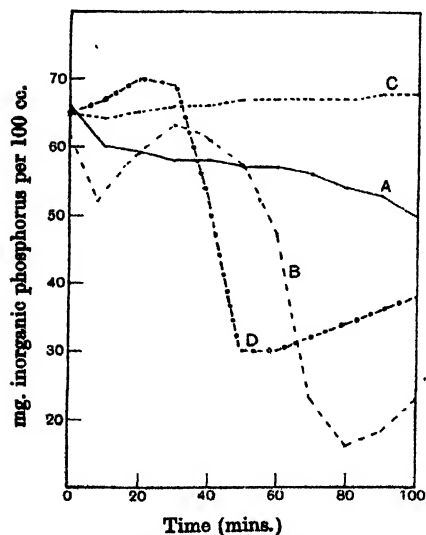


Fig. 5. Exp. 224.

- { A. Glycogen 0.9 %,  $p_H$  7.5.  
 { B. " 0.9 % with zymophosphate 1.5 %,  $p_H$  7.5.  
 { C. " 0.9 %,  $p_H$  6.5.  
 { D. " 0.9 % with zymophosphate 1.5 %,  $p_H$  6.5.

The results with 1.0 % glycogen alone and with 1.0 % zymophosphate with the extract of Exp. 241, are shown in the graphs of Figs. 6 and 7. It will be seen that the inhibition of the lactic acid formation by the zymophosphate comes decidedly into evidence after the first 10 minutes of the incubation and when the "extra ester accumulation" effect begins to appear. In this case the 1.0 % glycogen alone showed at the end of 90 minutes a production of 439 mg. lactic acid, the glycogen-zymophosphate mixture only 171 mg., a decrease in this case of 61 %. Although, in this case, the

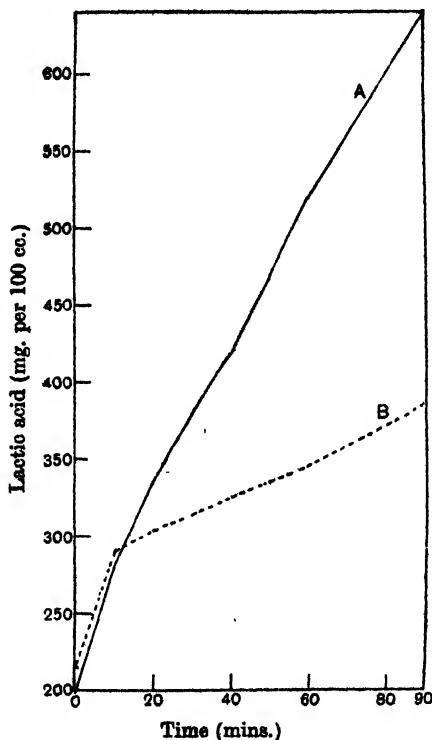


Fig. 6. Exp. 241. Lactic acid.

- A. Glycogen 1.0 % alone.  
B. Glycogen 1.0 % with zymophosphate 1.0 %.

inhibition of the lactic acid formation is related to the "extra ester accumulation," inhibition of the lactic acid production is not apparently always accompanied by any decided effect of the zymophosphate on the phosphorus changes in the controls.

In addition to the above observations the following was noted. Using 3 cc. of the trichloroacetic filtrates which were used for the inorganic phosphate estimations and adding 0.3 cc. of *N*/50 iodine, the reddish brown colour of the glycogen iodine compound is produced. By comparing in a comparator the colour of the corresponding samples of plain glycogen and of glycogen-zymophosphate mixture, a decided diminution in the strength of the colour

is readily detected in those cases where "extra ester accumulation" is produced to a decided extent by the zymophosphate. In addition, therefore, to the inhibition of the lactic acid formation, there occurs this phenomenon of more rapid loss of glycogen.

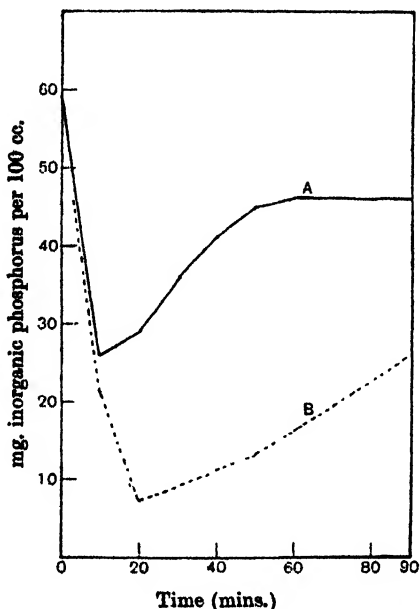


Fig. 7. Exp. 241. Inorganic phosphorus.

A. Glycogen 1.0 % alone.

B. Glycogen 1.0 % with zymophosphate 1.0 %

#### SUMMARY.

With the type of extract used the following has been noted. In a mixture of zymophosphate and glycogen in certain concentrations, there is less lactic acid produced than in the corresponding glycogen concentration in the absence of zymophosphate. This is associated, not invariably but very frequently, with an increased accumulation of phosphoric ester in the glycogen-zymophosphate mixture as compared with the corresponding glycogen experiment. This "extra ester accumulation," due to the presence of zymophosphate, is also associated with a more rapid loss of glycogen in addition to the above-mentioned inhibition of the lactic acid formation.

In concluding the author wishes to express his indebtedness to Bayer Products, Ltd., for a gift of 100 g. of pure sodium hexosediphosphate.

#### REFERENCE.

- \* Embden (1914). *Z. physiol. Chem.* 93, 1.

# CXI. LACTIC ACID FORMATION IN MUSCLE EXTRACTS.

## III. GLYCOLYSIS IN STERILE CELL-FREE EXTRACTS OF MUSCLE.

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(Received May 4th, 1928.)

SINCE the time of Claude Bernard, much investigation has been done on the subject of the "glycolytic enzyme" of blood and of muscle. The results of these investigations are very varied and even contradictory. One aspect of the subject, however, strikes one as worthy of further consideration. In the past, very few of the muscle extracts were bacteriologically sterile. It is certainly true that antiseptics were generally employed; but while on the one hand a negative result under these conditions proves nothing conclusive, since the antiseptic may be an inhibitor of the enzyme, on the other hand, a positive result, especially with an incubation period of more than three or four hours, makes one doubt the efficiency of the antiseptic. Whether a given antiseptic is an inhibitor of enzyme activity in the concentration required to prevent effectively the growth of microorganisms, or is an indifferent substance, cannot be demonstrated beyond doubt until the effect of its presence has been studied in an extract made sterile by some other method. If the problem of sterility is to be overlooked the period of incubation must not exceed three hours, and if in that period the loss of glucose is small, the results are not very convincing. The solution of this question lies in preparing extracts that are sterile, free from antiseptics and with a glycolytic activity of such magnitude that the loss of glucose is rapid. Such extracts have been prepared in this laboratory. The method consisted in filtering the muscle extract, previously frozen to a thick cream, through Berkefeld filter candles and then, at the end of the incubation, testing both for aerobic and anaerobic organisms by inoculation of a sample of the extract into Douglas broth incubated both aerobically and anaerobically.

This method of producing sterility was first applied to muscle extract by Brunton and Rhodes [1901]. They filtered an extract of sheep muscle through Pasteur-Chamberland filters: on keeping the filtrates in bottles plugged with cotton wool for several weeks, no bacterial growth was observed. The concentration of glucose fell from 1.5 to 0.75 % in 48 hours and this result was confirmed several times.

Since the experiments described in this paper were performed, Meyerhof [1926] also mentions that he was able to observe lactic acid formation from glycogen in extracts of muscle filtered through Berkefeld filters. His results were similar to those described here in that the first portion of the filtrate is inactive; but the experiments below were conducted with the intention of detecting destruction of glucose, not the breakdown of glycogen. It is in this sense that the term glycolysis is used here. In the investigator's experience it is more difficult to get an extract showing active glycolysis than one showing rapid lactic acid formation from glycogen. Glycolytic activity in this sense

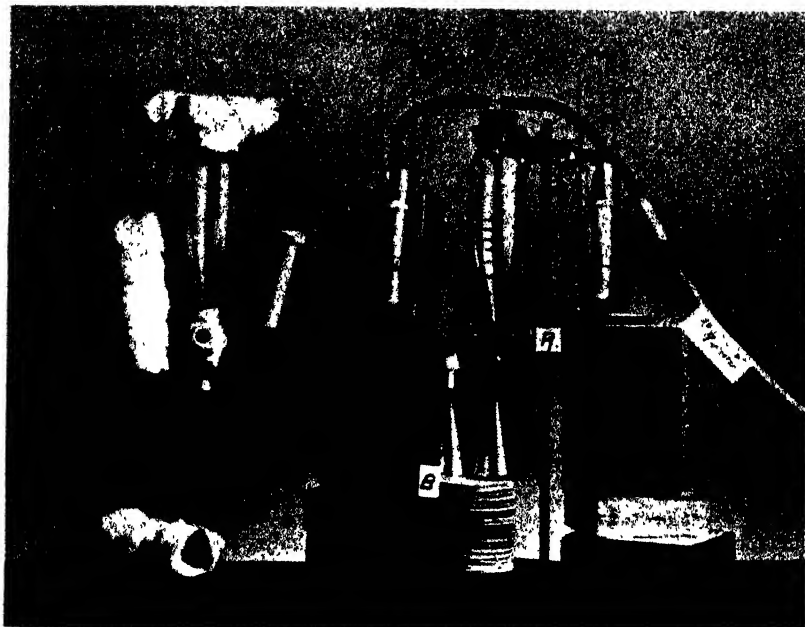


Fig. 1.

is a very uncertain phenomenon for some reason as yet obscure. Using certain precautions, however, it was possible in a great many cases, although not without many failures, to prepare sterile extracts of perfused rabbit and cat muscle, free from any intact cell structures, with a glycolytic activity of such a degree that the existence of the "glycolytic complex" in an active condition in cell-free extracts of muscle can no longer be considered doubtful.

#### *Preparation and filtration of the extracts.*

The animals were killed by a sharp blow on the head and immediately perfused with Ringer solution. The treatment thereafter was as is described in the first paper of this series, only in the cases described here the extracting

solution was one containing sodium bicarbonate and disodium hydrogen phosphate (hydrated) both to the extent of 0.5 %.

For the filtration the apparatus shown in Fig. 1 was employed. The two candles are joined by a T-piece fitted into a rubber stopper inserted into the top of the large collecting vessel known as a "Massen." The quickest filtration was obtained by reducing the pressure inside the "Massen" to about 100 mm. Hg and at the same time continually clearing the surface of the candles by the scrubbers as shown in the figure. These scrubbers consist of a ring of brass slightly wider than the diameter of the candle and lined on the inside with a strip of rubber pay-mat. By moving the scrubber up and down over the surface of the candle, the raised points of rubber clean the surface free of the slime that accumulates and chokes the filter.

For convenience the "Massen" was graduated to show every 50 cc. up to 300 cc. The collected filtrate was run out through the tap (A) into specially made conical flasks (B). The outlet tube from (A) is enclosed in a hood of wider bore; the conical flasks were therefore made with a long neck without any lip and of such a diameter that they could be pushed up inside the hood. The transference of the filtrate from the "Massen" to the conical flasks could thus be done without risk of infection, if the usual bacteriological precautions were taken.

The "Massen" and attached filters were sterilised by autoclaving at 120° for 20 minutes. The apparatus ready for sterilisation is shown on the left of Fig. 1. The tap (A) was tightly wrapped up in a pad of cotton wool, because when the suction is applied there often occurs at this tap a leak of air into the "Massen" in spite of the fact that the tap has been well greased by a mixture of white wax and lanolin. Since rubber shrinks on heating, the stopper with the T-piece tends to become loose and consequently was likewise covered up with a thick layer of cotton wool; it could thus be reinserted when the apparatus was cold without undue risk. When the required volume of the extract had been filtered, the glucose was introduced into the "Massen" via the tap (A). The tap bore was first cleared free of wax by a hot wire, the autoclaved glucose solution was introduced through the bore of the tap by means of a sterilised glass tube drawn out to a long thin point.

After shaking up, a few cc. were run out to wash out any glucose that might have adhered to the sides of the outlet tube. The extract was then measured out into the conical flasks, 25 cc. in each. One flask was taken for the initial values of glucose; the proteins were precipitated by tungstic acid and the glucose estimated by the method of Schaffer and Hartmann [1921]. The other samples were tested for sterility and analysed at the end of the incubation. For the detection of bacteria, two tubes of Douglas broth were each inoculated with about 1 cc. of the extract. One was incubated in the usual manner for aerobes. In the other the cotton wool plug was flamed and pushed in. The tube was fitted with a rubber stopper carrying a glass tube drawn out to a constriction ready for sealing: the air was sucked out and re-

placed by purified hydrogen; this alternate evacuating and refilling with hydrogen was done five times and lastly the tube was sealed when evacuated. This tube was incubated for the detection of anaerobes.

The difficulty of producing sterile filtrates was overcome after several failures but it was soon realised that all Berkefeld filters do not necessarily give sterile filtrates and unless one uses candles previously tested for their ability to hold back bacteria (in this case a peptone culture of *B. pyocyaneus* was used and the filtrate plated on agar), sterility is somewhat uncertain, and this entailed a great waste of effort. This difficulty was connected with the even greater one of producing extracts that were glycolytically active to a decided extent. Again after many failures the following details of technique had to be adopted.

The reaction of the extract must be on the alkaline side of  $p_H$  7.0; on the acid side of this the proteins do not pass through the pores of the filter, the candles choke very early in the filtration and the filtrates are inactive. This was the experience of Mudd [1922].

The extracts must be well frozen to a good stiff cream before the filtration is started so that the extract enters the filters in a concentrated condition by the freezing out of the water. Even with these precautions the best results were invariably obtained by using new, dry, but autoclaved filters. There was always however the attendant risk of infection under these conditions as the candles had not been previously tested. It was not possible to use new candles on every occasion. The most effective method of clearing an old filter was to fill it with 0.2 *N* sulphuric acid and to autoclave it in this condition. This produces a rapidly filtering candle, and in many cases this treatment could be repeated four or five times without the structure being destroyed or the ability to hold back bacteria being lost. Such filters, washed free of acid by bicarbonate and sucked "dry" on the aspirator, lose weight to the extent of 30 g. if heated at 100° until of constant weight. When using filters in this condition, i.e. with the kieselguhr skeleton damp, it was found that the first 100 cc. of the filtrate could be rejected because there was little or no glycolytic activity in this fraction; but the next 100 to 150 cc. usually showed glycolytic activity. With new, dry filters the whole of the filtrate could be used. The process of drying by heating to about 100° rapidly leads to the crumbling of the filter and for that reason it was given up; but, if the early portions of the filtrate were rejected, these old filters could be used again and again.

#### EXPERIMENTAL RESULTS.

Although no lactic acid estimations were made in this series given below, later work has indicated that the end product is lactic acid, a fact now well established. Never, in any single instance (and 34 cases were investigated for this) was any evidence found of the formation of a more complex carbohydrate (disaccharide) as suggested by Levene and Meyer [1912]. The glucose concentrations are expressed as mg. per 100 cc. extract.



Table of results.

No. of exp.	Glucose			Time hrs.	Remarks
	Start	End	Loss		
50 B	135	22	113	24	Tame rabbit
54 C	112	Trace	111	24	"
57 B	149	"	148	24	"
59 B	157	25	132	24	"
60 C	136	Trace	135	24	"
73 B	133	"	132	4	"
77 B	154	12	142	4	Cat
79	169	169	0	3	1st 50 cc. Tame rabbit
	182	173	9	3	2nd 50 "
	173	98	75	3	3rd 50 " Damp filters
	187	55	132	3	4th 50 "
82	147	71	76	2	1st 50 cc. Tame rabbit
	146	19	127	2	2nd 50 "
	155	24	131	2	3rd 50 " New dry filters
	146	88	58	2	4th 50 "
	152	129	23	2	5th 50 "
83	254	243	11	2	1st 50 cc. Tame rabbit
	285	243	42	2	2nd 50 "
	268	177	91	2	3rd 50 "
	258	197	61	2	4th 50 " Damp filters
	251	120	131	2	5th 50 "
86	308	113	195	3	1st 100 cc. Tame rabbit
	332	103	229	3	2nd 100 "
87	512	319	132	3	1st 100 cc. Tame rabbit
89	351	21	330	8	Tame rabbit
91	641	402	239	3	"
93	574	407	167	3	Cat
95	332	152	180	2	"
96	336	119	217	3	Two tame rabbits
97	355	179	176	3	Cat
99	330	174	156	2	1st 50 cc. Two tame rabbits
	345	170	175	2	2nd 50 "
	327	122	205	2	3rd 50 "
	335	216	119	2	4th 50 " New dry filters
	343	279	64	2	5th 50 "
100	345	129	216	2	Two tame rabbits
102	308	233	75	1	1st 75 cc. Cat
	305	152	153	2	2nd 75 " New dry filters
	291	120	171	2	3rd 75 "
	291	38	253	2	" " plus 0.1 g. of NaHCO <sub>3</sub>

Quoted above are 21 cases out of a total of 102 extracts filtered. The 81 cases not recorded include both those that were infected and those that showed little or no loss of glucose. The latter are interesting although not of direct value in a positive sense. Many of the early failures were due to the use of an unsuitable extracting solution. The results showed that a mixture of sodium or potassium bicarbonate with the chlorides or dibasic phosphates of those metals was quite suitable if the salts were used in concentrations not greater than about 1 %. A solution of 5 % Na<sub>2</sub>HPO<sub>4</sub> · 12H<sub>2</sub>O gave an extract that coagulated during the filtration. The extraction of the muscle proteins was very extensive with this solution but that very fact rendered it highly unsuitable for filtration purposes.

Another source of failure, which was not appreciated until experiments had been done with unfiltered extracts, is that tame rabbits are not, for some peculiar reason, the best animals for the production of very glycolytic extracts.

During the course of the author's investigations on glycolysis, over 200 different extracts have been examined and the best animals for this type of investigation have been found to be either wild rabbits or cats. Even with these animals one occasionally encounters extracts of apparently normal individuals that show only a slight glycolytic activity, no matter how rigorously a standardised method of preparation is employed. In the case of tame rabbits, extracts prepared in the above manner show good glycolysis in only about 50 % of the cases. In this respect lactic acid formation from glucose must be distinguished from lactic acid formation from glycogen, the latter unlike the former takes place at a fairly uniform rate in all freshly prepared extracts. This is still under investigation.

#### DISCUSSION.

The above results indicate that the glycolytic enzyme (or enzymes) can be demonstrated by the destruction of added glucose in filtrates from Berkefeld filters. No activator of any description, such as the pancreatic extract employed by Cohnheim [1903; 1904, 1, 2; 1907], was added. More important still, no intact cell structures were present, and the condition put forward by Fletcher [1911], that glycolysis can only take place if the muscle structure is preserved, is not indicated here at all. If the above type of extract be centrifuged in a Sharples supercentrifuge, a clear transparent solution is produced, similar to the filtrates from the Berkefeld filters, but of course not sterile. The removal, in this manner, of the muscle debris that passes through the mesh of the muslin used in the hand-press, has absolutely no effect on the glycolytic activity. The muscle enzymes are in solution in the same sense as the muscle proteins, although removed from the internal environment of the muscle fibres.

The question of sterility is not, in the author's opinion, an important one for the following reason. An examination of the literature of this subject gives one the impression that an incubation period of at least 24 hours is necessary before a decided loss of glucose can be detected; but an extensive examination of many extracts at intervals of 10 minutes over a period up to 2 hours, indicated that the greatest rate of destruction of glucose takes place in the first 10 minutes of the incubation. In extracts of cat muscle the loss of glucose in the first 10 minutes has been observed to be as high as 78 mg. per 100 cc. extract: with wild rabbit muscle it has been as high as 95 mg. This high rate lasts for about 40 minutes; consequently the period of incubation need not exceed, at the longest, 2 hours, and even 90 minutes is quite sufficient. In that time the effect of bacterial growth is not evidenced. The many cases of unfiltered extracts, prepared with no precautions against infection and showing no loss of glucose at the end of a 2 hours' incubation, indicate that it is the muscle enzymes and not bacteria that give rise to lactic acid in the early period of the incubation, and it is in that early period that the most important and interesting phase of lactic acid formation takes place.

## SUMMARY.

Glycolysis has been observed in extracts of perfused muscle after filtration through Berkefeld filters. The results quoted are with those filtrates that were bacteriologically sterile. The original observation of Brunton and Rhodes on glycolysis in similar filtrates has been confirmed.

In concluding, the author wishes to express his gratitude to Prof. E. Waymouth Reid, F.R.S., who suggested and supervised this research; likewise his thanks to Mr W. Milne for taking charge of the sterilising and care of the apparatus, and also to Prof. W. J. Tulloch, for his advice on the bacteriological side of the problem.

## REFERENCES.

- Brunton and Rhodes (1901). *Proc. Roy. Soc. Lond.* **68**, 417.  
Cohnheim (1903). *Z. physiol. Chem.* **39**, 396.  
—— (1904, 1). *Z. physiol. Chem.* **42**, 411.  
—— (1904, 2). *Z. physiol. Chem.* **43**, 547.  
—— (1907). *Z. physiol. Chem.* **47**, 253.  
Fletcher (1911). *J. Physiol.* **43**, 286.  
Levene and Meyer (1912). *J. Biol. Chem.* **11**, 353.  
Meyerhof (1926). *Biochem. Z.* **178**, 417.  
Mudd (1922). *Amer. J. Physiol.* **63**, 429.  
Schaffer and Hartmann (1921). *J. Biol. Chem.* **45**, 377.

## CXII. HAEMOCYANIN.

### PART V. THE OXYGEN DISSOCIATION CURVE OF HAEMOCYANIN FROM THE SNAIL (*HELIX POMATIA*) IN DIALYSED SOLUTION.

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*(Received June 19th, 1928.)*

THE determination by the authors [1927] of the isoelectric points of the haemocyanins from certain species conclusively demonstrated, in confirmation of the provisional results described in their earlier communications [1926, 1, 2], that the  $[H']$  at which the affinity for oxygen of haemocyanin from the crab (*Cancer pagurus*) passed through a minimum did not coincide with the isoelectric point of the protein but was, in fact, considerably on the alkaline side of this point. At the same time, a relation was found to exist between the affinity for oxygen of the pigment from this species and the viscosity of its solutions, the  $p_H$ -affinity curve being antibatic to the  $p_H$ -viscosity curve, and the  $p_H$  corresponding with minimal affinity practically coinciding with that at which the viscosity of the solution passed through a maximum. This relation was established only in the case of haemocyanin from *Cancer*. Nevertheless, an antibasis between viscosity and affinity was also observed with haemocyanin from the lobster (*Homarus vulgaris*); the viscosity appeared, moreover, to be tending towards a maximum, and the affinity towards a minimum, at a  $p_H$  which was on the alkaline side of the isoelectric point, but which could not be reached experimentally owing to the slight solubility of the pigment at this particular acidity. It therefore seemed probable that the relation was a general one, and the authors predicted that the affinity of haemocyanin from the snail (*Helix pomatia*), the viscosity of solutions of which was shown to pass through a maximum at about  $p_H$  8.5, would be found to be at a minimum at this acidity. Shortly after the writers had sent their paper to press, a publication by Hogben and Pinhey [1926] came to their notice in which oxygen dissociation curves of the haemocyanin from *Helix* were investigated over a  $p_H$  range of 6.8 to 9.05 using the colorimetric method of Pantin and Hogben [1925]. The resulting curves are similar to those obtained with haemocyanin from *Cancer*, and Hogben and Pinhey comment upon them as follows: "Accepting these values (i.e. the values of  $x_{50}$ , the oxygen partial

pressures corresponding with 50 % saturation), which are consonant with conclusions derived from four different sets of experiments, it would seem that, slight as is the effect compared with the case of crustacean haemocyanin, the haemocyanin of *Helix* exhibits the same fundamental relation to hydrogen ion concentration as the latter, namely, that the effect of increasing hydrogen ion concentration up to a certain point is to diminish the affinity for oxygen, and beyond this point to increase it. The point of minimal value for  $\alpha_{50}$  corresponds to a  $p_H$  of  $8.0 \pm 0.1$ . That this conclusion is justified in spite of the small order of the effect is, however, quite conclusively reinforced from another consideration. The dissociation curves obtained on the acid side of the critical  $p_H$  referred to are not of the same shape as those obtained on the alkaline side. They are flatter at the top, that is to say, they show relatively less affinity for oxygen at high tensions than the alkaline curves passing through the same points at lower tensions." The critical  $p_H$  ( $8.0 \pm 0.1$ ) of these authors, although not identical with the  $p_H$  (8.5) at maximal viscosity, corresponds with it very closely and hence appears to fulfil the prediction mentioned above. Despite this, however, it has been considered advisable, for a number of reasons, to examine the effect of  $p_H$  on the oxygen dissociation curve of haemocyanin from *Helix* by the gasometric method. The results, which are presented below, are not in agreement with those obtained by Hogben and Pinhey, and give no indication of a relationship between affinity and viscosity such as exists in the case of haemocyanin from *Cancer*.

#### EXPERIMENTAL METHODS.

The serum employed was concentrated and dialysed according to the procedure outlined in earlier communications. Adjustments in  $p_H$  were effected by addition of alkali or acid to the dialysed serum, these reagents being employed only on the alkaline or acid sides respectively of the isoelectric point of the haemocyanin in question. Where necessary, the protein was brought from the alkaline to the acid side of the isoelectric point by the method previously described [1926, 2]. All determinations of  $p_H$  were made electrometrically under the conditions used throughout this series of investigations.

#### *Determination of the oxygen content of solutions of haemocyanin.*

In the determinations of the oxygen contents of solutions of haemocyanin from *Cancer* by means of the Van Slyke apparatus [1926, 2], mercuric cyanide was used to facilitate the liberation of oxygen. With haemocyanin from *Helix*, which possesses a much greater affinity for oxygen than that from *Cancer*, especially at low pressures of this gas, the mercuric cyanide reagent by no means completely displaces the oxygen from its combination with the protein, and hence another reagent has been employed. In the choice of this reagent use has been made of the observation of Redfield, Coolidge and Hurd [1926] that potassium cyanide, when used in conjunction with the Van Slyke

apparatus, quantitatively displaces the combined oxygen from *Limulus* blood. There are, however, certain objections to the use of this reagent. Redfield, Coolidge and Montgomery [1928] have recently pointed out that a small quantity of the oxygen combined with the haemocyanin is converted by the potassium cyanide into a form which is no longer liberated on evacuation, although they have succeeded, by suitably modifying their technique, in almost completely suppressing this loss. The strongly alkaline reaction of solutions of potassium cyanide is also a disadvantage, since, under some circumstances, it would cause coagulation of the protein. It seemed probable that these difficulties might to some extent be overcome by using potassium mercuric cyanide in place of potassium cyanide. The former substance has therefore been used to displace oxygen in combination with haemocyanin in all the experiments carried out with the pigment from *Helix*. The technique employed is as follows. 5 cc. of the haemocyanin solution, brought into equilibrium with the gas mixture in the manner previously described, is drawn from an Ostwald pipette into the Van Slyke apparatus and washed in with 2 cc. of water which has been previously evacuated and displaced into the cup of the apparatus. One drop of octyl alcohol is then introduced into the pipette, after which the mercury is lowered and the gases extracted from the solution as completely as possible by shaking for 1 minute. One cc. of a gas-free 1 % solution of potassium mercuric cyanide is now added and the shaking resumed and continued for 3 minutes. At the end of this period, the extracted solution is removed from the apparatus through the trap and the gases analysed in the manner hitherto adopted in this work. The volume (1 cc.) of the 1 % solution of potassium mercuric cyanide used in each estimation has been found sufficient to prevent any recombination of the haemocyanin with oxygen, as shown by the development of blue colour on its coming into contact with the air on removal from the extraction chamber.

That potassium mercuric cyanide resembles potassium cyanide in its action in displacing oxygen from its combination with haemocyanin is doubtless due to the fact that, in its aqueous solutions, it is to some extent dissociated into mercuric and potassium cyanides. Corresponding with this, it is less efficient, in the sense that a greater concentration is required to produce the same effect, than potassium cyanide, and it is consequently uncertain if it possesses any advantages over the latter reagent. It was employed in these experiments, however, because it was thought that its efficiency, although apparently smaller than that of potassium cyanide, would, if referred to the amount of free potassium cyanide which it develops in solution, be actually greater, since the undissociated potassium mercuric cyanide would form a potential supply of potassium cyanide, and hence would resemble the latter in its action on oxyhaemocyanin without possessing the disadvantages, mentioned above, associated with the presence in the solution of moderate concentrations of free potassium cyanide. An experiment was carried out with haemocyanin from *Helix* to test the relative efficiencies of mercuric cyanide, potassium cyanide

and potassium mercuric cyanide. The solution of haemocyanin was brought into equilibrium with air at a temperature of  $23^{\circ}$ , and estimations were made of the oxygen extracted from a 5 cc. sample by the Van Slyke apparatus (1) in the absence of any cyanide reagent, (2) with the addition of 2 cc. of a saturated solution of mercuric cyanide, (3) with the addition of 0.5 cc. of a 1 % solution of potassium cyanide, and (4) with the addition of 1 cc. of a 1 % solution of potassium mercuric cyanide; it had been previously ascertained that the amounts of cyanide reagent used in the last two estimations were sufficient to decolorise the haemocyanin completely even when in contact with the atmosphere. The manometer readings, which represent the partial pressure of the oxygen in the extracted gases when the latter occupied a volume of 0.494 cc., were 103.5, 107.5, 143.5 and 146.5 mm. respectively. This experiment shows that the effect of the mercuric cyanide is of too small a magnitude for this reagent to be of assistance in connection with estimations carried out with haemocyanin from *Helix*. The values obtained with potassium cyanide and potassium mercuric cyanide agree fairly closely although a slightly greater yield of oxygen has been obtained with the latter reagent. While the difference between the results obtained in these two experiments is greater than the normal experimental error, it is impossible on the basis of this one set of estimations to claim for potassium mercuric cyanide any superiority over potassium cyanide, although such superiority is certainly suggested.

An interesting deduction may be made from the results just described. When the oxygen content of the haemocyanin solution is calculated in the usual manner from the manometer readings obtained in Exps. 1 and 4, the values of 1.266 and 1.794 are obtained respectively. The difference between these values, namely 0.528, clearly represents the vols. % of oxygen which, in Exp. 1, remained in combination with the haemocyanin under the conditions existing in the Van Slyke apparatus immediately prior to the removal of the sample. Now, the total amount of oxygen in combination with the haemocyanin when in equilibrium with air at a temperature of  $23^{\circ}$  is  $(1.794 - 0.590)$  vols. %, 0.590 vols. % being the amount of oxygen physically dissolved in the solution, and it will be shown later that the haemocyanin is 97.5 % saturated under these conditions. It follows that the maximum amount of oxygen with which this particular solution is capable of combining chemically is 1.235 vols. %, and hence in Exp. 1 the haemocyanin was 42.8 % saturated when removed from the Van Slyke apparatus. The oxygen partial pressure with which this solution was in equilibrium in the pipette of the apparatus, when calculated on the lines indicated in Part II [1926, 1], is found to be about 1.5 mm., and the temperature of the apparatus was  $18^{\circ}$ . Hence, haemocyanin from *Helix* is 42.8 % saturated with oxygen when in equilibrium with a partial pressure of oxygen of 1.5 mm. and at a temperature of  $18^{\circ}$ . This figure is probably only a rough one, but it serves to illustrate the great affinity for oxygen of haemocyanin from *Helix*. At a temperature of  $23^{\circ}$ , as will be seen from Fig. 4, the haemocyanin is about 26 % saturated at the same oxygen partial pressure.

## RESULTS.

*Influence of haemocyanin on the solubility of oxygen in water.*

One of the greatest disadvantages attached to the gasometric method for the determination of the oxygen content of solutions of haemocyanin is the uncertainty which exists regarding the influence, if any, which the haemocyanin exerts upon the solubility of oxygen in water. In Part 1 [1925] of this series, in which the oxygen dissociation curves of the haemocyanin in the sera of four species of decapod crustacea were determined, the amount of oxygen physically dissolved was ascertained by determining, for each specimen of serum examined, the oxygen content of samples equilibrated with increasingly greater oxygen partial pressures until proportionality was obtained between oxygen content and oxygen pressure, and then assuming the applicability of Henry's law. The solubility curves so obtained, while approximating to that for sea-water, indicated, in each case, absorption coefficients which were slightly but definitely smaller than that of sea-water. Assuming that the salt content of the blood was identical with that of sea-water, an assumption for which there is some justification, one could legitimately conclude that haemocyanin depresses the solubility of oxygen in water. When, however, the same method was employed under better conditions and with improved technique in connection with the determination of the oxygen dissociation curves of haemocyanin in salt-free solutions at different  $[H']$  [1926, 1, 2], it was found that the results could be best interpreted by consistently assuming an oxygen solubility identical with that of distilled water. The haemocyanin appeared to exert no appreciable influence on the solubility of oxygen. Similar results have been obtained with haemocyanin from *Helix* in the present investigation. On the other hand, Redfield, Coolidge and Hurd [1926], using the same principle, found that the haemocyanin in *Limulus* serum depressed the oxygen solubility, although they admit that their figures "are subject to an error, which amounts to a loss of about 3 to 10 % of the oxygen present in the sample." In a recent paper Redfield, Coolidge and Montgomery [1928] have directly determined the absorption coefficient for oxygen of *Limulus* serum. The value which they obtain is smaller than that for sea-water by about 10 %, and they therefore consider that the haemocyanin in *Limulus* blood depresses the solubility of oxygen by about this amount. In the present investigation the solubility of oxygen in dialysed *Helix* serum has been determined by the method due to the above authors, with the modification that potassium mercuric cyanide has been used in place of the potassium cyanide. The cyanide reagent was evacuated in the Van Slyke apparatus before addition to the serum, and the latter was subsequently evacuated in the high vacuum produced by a rotatory oil pump. It was then equilibrated with air (bar. 761 mm.) at 23° and the oxygen content determined in the usual manner. The values



obtained in three estimations on the same sample were 0.610, 0.599 and 0.593 vols. % respectively. Calculating from the mean of these values the vols. % of oxygen dissolved by the serum at an oxygen partial pressure of 159 mm., a value of 0.615 is obtained, which is in satisfactory agreement with the value of 0.620 for distilled water. Calculations of the volume of oxygen physically dissolved by haemocyanin solutions have accordingly been made on this basis. While it is conceivable that the pigment does actually cause a slight depression in the solubility of oxygen, these results indicate that this depression is of such small magnitude that the error, if any, introduced by neglecting it is not sufficiently great to impair the conclusions finally reached. It should be pointed out that this result does not necessarily conflict with that of Redfield and his co-workers, for the haemocyanins from *Helix* and *Limulus* are different proteins and may behave differently with respect to their influence on oxygen solubility.

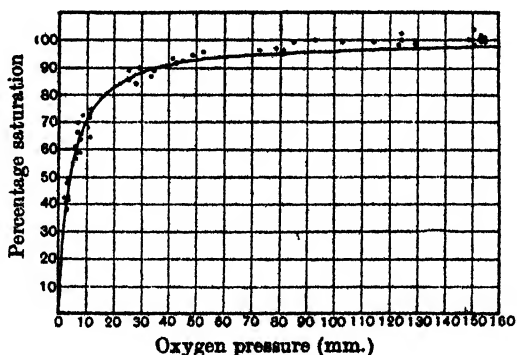


Fig. 1.

*Influence of  $p_H$  on the oxygen dissociation curve of haemocyanin from Helix.*

The oxygen dissociation curve of haemocyanin from *Helix* has been examined at a temperature of 23° and over a  $p_H$  range of from 4.04 to 9.04. The data are given in Table I. In column 4 the values for the oxygen physically dissolved in the solution have been calculated on the basis outlined above. By subtracting the figures in column 4 from the corresponding figures in column 3, the values for the combined oxygen (column 5) have been obtained. From these the percentage saturation (column 6) of the haemocyanin for each oxygen partial pressure has been calculated, using the figure given on the last line of each series of experiments as representing the maximum amount of oxygen with which the haemocyanin is capable of combining. The significance of column 7 will be pointed out below.

These results have been plotted in Fig. 1, from which it is evident that the points lie about one smooth curve. Haemocyanin from *Helix* thus differs from the pigments from *Cancer* and *Homarus* in that, in the absence of salts, its

Table I.

<i>P<sub>H</sub></i>	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation	Percentage saturation (corrected)
4.04	2.89	0.436	0.011	0.425	49.1	47.9
	8.38	0.657	0.033	0.624	72.1	70.3
	11.61	0.687	0.045	0.642	74.2	72.4
	33.38	0.878	0.130	0.748	86.5	84.3
	81.84	1.147	0.319	0.828	95.7	93.3
	128.61	1.363	0.502	0.861	99.5	97.0
	153.62	1.453	0.599	0.854	98.7	96.2
	153.62	1.475	0.599	0.876	101.3	98.7
	—	—	—	0.865	100.0	97.5
	—	—	—	—	—	—
4.79	2.74	0.434	0.011	0.423	41.5	40.4
	6.73	0.736	0.026	0.710	69.6	67.9
	29.34	1.032	0.115	0.917	89.9	87.7
	78.92	1.300	0.308	0.992	97.3	94.8
	129.75	1.509	0.506	1.003	98.3	95.9
	154.87	1.613	0.604	1.009	98.9	96.3
	154.87	1.633	0.604	1.029	100.9	98.2
	212.00	1.852	0.827	1.025	100.5	98.0
	—	—	—	1.020	100.0	97.5
	—	—	—	—	—	—
6.25	2.85	0.729	0.010	0.719	47.9	46.7
	6.45	1.017	0.025	0.992	66.1	64.5
	11.13	1.132	0.043	1.089	72.6	70.8
	25.24	1.439	0.098	1.341	89.4	87.2
	72.60	1.724	0.283	1.441	96.1	93.7
	114.21	1.936	0.445	1.491	99.4	96.9
	151.06	2.071	0.589	1.482	98.2	96.3
	151.06	2.143	0.589	1.554	103.6	101.1
	—	—	—	1.500	100.0	97.5
	—	—	—	—	—	—
6.35	3.79	0.629	0.015	0.614	49.1	47.9
	11.30	0.846	0.044	0.802	64.2	62.6
	27.65	1.161	0.108	1.053	84.2	82.1
	49.25	1.369	0.192	1.177	94.2	91.8
	102.67	1.642	0.400	1.242	99.4	96.9
	123.62	1.708	0.482	1.226	98.1	95.6
	150.48	1.837	0.587	1.250	100.0	97.5
	—	—	—	1.250	100.0	97.5
	—	—	—	—	—	—
	—	—	—	—	—	—
7.81	2.83	0.485	0.011	0.474	40.8	39.8
	7.00	0.707	0.027	0.680	58.5	57.1
	7.96	0.780	0.031	0.749	64.5	62.8
	11.10	0.878	0.043	0.835	71.9	70.1
	41.31	1.244	0.161	1.083	93.2	90.9
	53.14	1.318	0.207	1.111	95.6	93.2
	93.51	1.527	0.365	1.162	100.0	97.5
	151.05	1.751	0.589	1.162	100.0	97.5
	—	—	—	1.162	100.0	97.5
	—	—	—	—	—	—
8.74	2.90	0.450	0.011	0.439	41.6	40.6
	6.15	0.664	0.024	0.640	60.7	59.1
	10.04	0.761	0.039	0.712	67.5	65.8
	34.45	1.072	0.134	0.938	88.9	86.7
	42.88	1.135	0.167	0.968	91.8	89.5
	81.61	1.322	0.318	1.004	95.2	92.8
	124.24	1.540	0.485	1.055	100.0	97.5
	153.20	1.652	0.597	1.055	100.0	97.5
	—	—	—	1.055	100.0	97.5
	—	—	—	—	—	—
9.04	2.50	0.431	0.010	0.421	38.0	37.0
	6.00	0.655	0.023	0.632	56.7	55.6
	7.51	0.735	0.029	0.706	63.7	62.1
	25.17	1.050	0.098	0.952	85.9	83.8
	45.33	1.200	0.177	1.023	92.3	90.0
	84.92	1.431	0.331	1.100	99.3	96.8
	124.12	1.620	0.484	1.136	102.5	100.0
	149.79	1.692	0.584	1.108	100.0	97.5
	—	—	—	1.108	100.0	97.5
	—	—	—	—	—	—

oxygen dissociation curve undergoes no detectable change with changes in  $p_H$ . The curve is, moreover, different in shape from those obtained with crustacean haemocyanins: it rises steeply from the origin and exhibits no point of inflexion. Superficially, it is of the hyperbolic type which would be expected if the dissociation of the haemocyanin were a unimolecular reaction following the mass law in the form in which it is applicable to homogeneous solutions. If such were the case, and the reaction could be represented by the equation



then

$$\frac{[\text{HcyO}_2]}{[\text{Hcy}][\text{O}_2]} = K \quad \text{.....(2)}$$

in which  $K$  is the equilibrium constant of the reaction. By putting  $[\text{Hcy}] = y$ ,  $[\text{HcyO}_2]$  becomes  $1 - y$ ; since, further,  $[\text{O}_2] \propto p$ , where  $p$  is the partial pressure of oxygen, equation (2) can be written in the form

$$\frac{1-y}{y} = kp \quad \text{.....(3)}$$

or,

$$\log \frac{1-y}{y} = \log k + \log p \quad \text{.....(4)}$$

from which it follows that, provided equation (1) correctly represents the reaction in question, a straight line inclined to the axes at an angle of  $45^\circ$

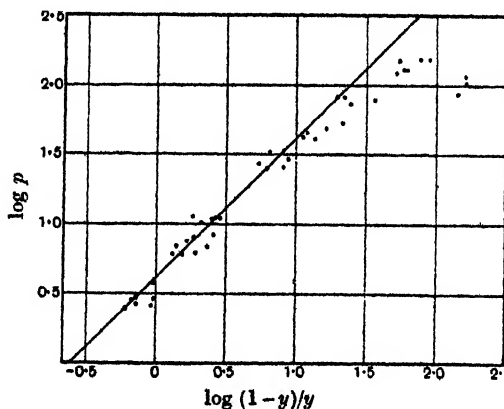


Fig. 2.

should be obtained by plotting  $\log (1 - y)/y$  against  $\log p$ . The values of these expressions have been calculated from the figures in Table I and then plotted in Fig. 2. With the exception of those corresponding with high oxygen partial pressures, the points fall satisfactorily about a straight line inclined to the axes at an angle of  $45^\circ$ . It therefore appears that the dissociation of oxyhaemocyanin is correctly represented by equation (1) and it is of interest to construct a curve fulfilling the requirements of this equation and at the same time corresponding as closely as possible with the experimentally determined values. Now, the abscissa of the point at which the straight line in Fig. 2 cuts the axis represents the value of  $\log k$  in equation (4); hence, reading this value from Fig. 2,  $\log k = -0.61$ ; consequently  $k = 0.245$ . Substituting this value

for  $k$  in equation (3), the value of  $y$ , and hence the percentage saturation of the haemocyanin, can be calculated for any value of  $p$ . The curve drawn in Fig. 1 is the theoretical curve which has actually been constructed by this method using the above numerical value of  $k$ , and the experimentally determined values are, considering the difficulties attached to the estimations, in excellent agreement with it. The greatest consistent divergence occurs at high oxygen pressures. This, however, is not surprising when the method employed for determining the saturation value of the haemocyanin is recalled. For this purpose, it has been assumed that the haemocyanin was saturated with oxygen when the oxygen content was, within the limits of experimental error, proportional to the oxygen partial pressure with which the solution was in equilibrium; the saturation value has then been obtained from the total oxygen content by deducting the amount of oxygen physically dissolved by the solution. Now, if the combination between the haemocyanin and the oxygen follows the law of mass action in the sense indicated above, the haemocyanin must approach saturation asymptotically, as does the theoretical curve drawn in Fig. 1, and it is clear that the increment produced in the amount of oxygen combined with the haemocyanin by increasing the oxygen partial pressure by a relatively large amount will, at the higher pressures employed in these experiments, be so small as to fall within the limits of experimental error. The discrepancy between the upper portion of the curve in Fig. 1 and the experimentally determined points could readily be accounted for in this way, quite apart from the possibility that the solubility of oxygen in the solutions is slightly smaller than has been assumed, a possibility which, if substantiated, would also tend to diminish the discrepancy mentioned above. These considerations indicate that the degree of saturation of the haemocyanin at oxygen partial pressures of from about 70 mm. upwards is more accurately expressed by the theoretical curve than by the actual values determined experimentally, and it would therefore seem that, under the conditions employed in these experiments, haemocyanin from *Helix* is about 97.5 % saturated with oxygen when in equilibrium with an oxygen partial pressure of about 150 mm., i.e. when in equilibrium with air. The figures given in column 6 of Table I have accordingly been re-calculated on this basis, the revised values for percentage saturation so obtained being given in the last column of the same table. Fig. 2 has been re-drawn in Fig. 3 from these revised figures, and from this a value of  $k = 0.224$  has been obtained. With this new value of  $k$  the theoretical dissociation curve shown in Fig. 4 has been constructed; the revised experimental values have also been plotted in this figure.

#### DISCUSSION.

In view of the history of the investigation of the oxygen dissociation curve of haemoglobin there will necessarily be some hesitation in accepting the implications of the results of the foregoing experiments. Nevertheless, the authors feel that the values obtained for the percentage saturation of haemo-

cyanin from *Helix* at different oxygen partial pressures are, as an inspection of Fig. 4 will show, in such satisfactory agreement with the values calculated from equation (1) on the assumption that the law of mass action is applicable to the system under consideration, that there can be little doubt that the dissociation of the oxyhaemocyanin forms an example of a microheterogeneous

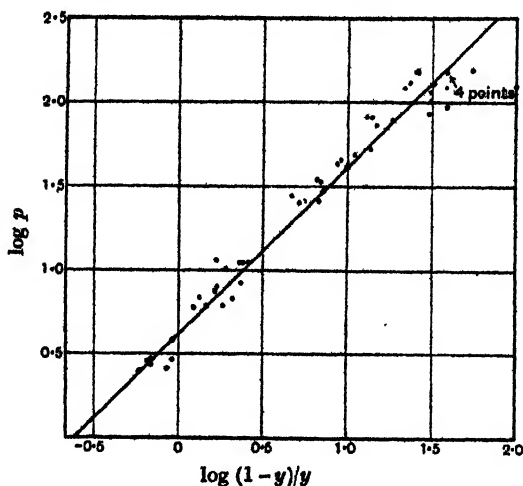


Fig. 3.

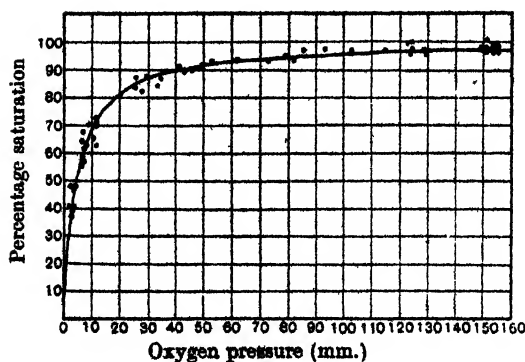


Fig. 4.

reaction which takes place according to the laws which have been developed for reactions occurring in homogeneous systems.

The theoretical curve in Fig. 4 has been deduced on the assumption that, in the formation of oxyhaemocyanin, one molecule of haemocyanin reacts with one molecule of oxygen, and, on any other simple assumption, a curve different from that shown would be obtained.

Now, Begemann [1924] has shown that the haemocyanins from *Helix pomatia* and *Carcinus maenas*, when saturated with oxygen, are in combination

with an amount of this element which corresponds to one atom of oxygen for each atom of copper contained in the haemocyanin, while, more recently, Redfield, Coolidge and Montgomery [1928] have examined the haemocyanins from a large number of species and have found that in every case the same relation exists. The work of the latter authors is of such an extensive and convincing nature that it cannot be doubted that the haemocyanins, in general, combine with oxygen in virtue of the copper which they contain and do so, moreover, in the stoichiometrical proportions indicated above. It might be mentioned here that the present authors in some unpublished experiments of a preliminary nature have themselves found that the same ratio between copper content and oxygen capacity exists in the case of haemocyanin from *Cancer pagurus*. These results, taken in conjunction with the fact, established above, that haemocyanin from *Helix* combines with oxygen according to equation (1), indicate that each molecule of the haemocyanin from this particular species contains two atoms of copper. In this connection, it is interesting to note that Redfield, Coolidge and Shotts [1928] have, from the results of their determination of the copper content of haemocyanin from *Limulus* combined with the value of the molecular weight of this protein as determined by Cohn [1925], reached an identical conclusion with respect to the pigment from this species.

The deduction of the curve drawn in Fig. 4 by application of the law of mass action to equation (1) involves the further assumption that the haemocyanin in question is molecularly dispersed in its solutions, although these are of a colloidal nature, and the correspondence between the curve so deduced and the values determined experimentally points to the truth of this assumption. This result, which, it should be emphasised, has been obtained with salt-free solutions and hence does not necessarily hold for solutions containing salts, is of some interest in a general way. Thus, it has been shown above that change in  $p_H$  produces no detectable change in the shape of the dissociation curve; it follows that the degree of dispersion of the pigment must remain constant throughout the range of acidity investigated. Nevertheless, the dialysed solutions of haemocyanin from *Helix* employed in these experiments differed markedly in appearance according to the  $p_H$  of the solution. In the region of the isoelectric point ( $p_H$  5.3) the solutions exhibited a strong opalescence (Tyndall phenomenon) which was less marked at  $[H^+]$  more removed from this point, and, in the more alkaline solutions employed, was scarcely apparent at all. It follows, since the haemocyanin was throughout in a state of molecular dispersion, that these changes were due, not to changes in the degree of aggregation of the micellae, but to changes in their state of hydration.

The most interesting aspect of these experiments is, however, the marked contrast which is shown to exist between the oxygen dissociation curve of haemocyanin from *Helix* and the curves from the crustacean haemocyanins. The behaviour of, for example, the pigment from *Cancer* differs from that of haemocyanin from *Helix* in two important respects: its affinity for oxygen depends upon the reaction of the solution, while its dissociation curve is, under

certain conditions of acidity, of an S-shape. If the shape of the oxygen dissociation curve of haemocyanin from *Helix* may be interpreted, as has been done above, as indicating that the pigment is molecularly dispersed in solution, it would appear probable that the S-shape of the dissociation curves of other haemocyanins is due primarily to aggregation of the molecules. That the molecules are present in the form of aggregates is, moreover, suggested by other considerations. Thus, if it be assumed that the degree of aggregation is  $n$  and that the aggregates are not appreciably hydrated and hence occupy a relatively small volume of the solution, then the effective concentration of the reduced haemocyanin, i.e. the concentration controlling the rate at which it combines with oxygen when the whole of the pigment is in the reduced form, would be  $y/n$ , where  $y$  is the actual concentration, and would remain practically constant at this value until  $n - 1$  molecules in each aggregate had been converted into the oxidised form. On the other hand, the effective concentration of the oxyhaemocyanin would be identical with the actual concentration: aggregation would not influence the rate at which the oxyhaemocyanin dissociated into oxygen and the reduced pigment. It follows that the percentage saturation of the haemocyanin with oxygen would be practically proportional to the oxygen partial pressure with which the solution was in equilibrium until  $n - 1$  molecules in each aggregate had been converted into oxyhaemocyanin. In other words, the initial portion of the oxygen dissociation curve should be a straight line passing through the origin. As pointed out in Part IV [1927], this is actually the case with haemocyanin from *Cancer* at acidities in the neighbourhood of the isoelectric point, and this seems to indicate the correctness of the above reasoning. At acidities more removed from, and on the alkaline side of, the isoelectric point, an S-shaped curve is obtained, which presumably results from the combined effect of aggregation and other factors such as hydration of the micellae. Why the affinity for oxygen of haemocyanin from *Cancer* should be much smaller when it is present as anion than when it is in the undissociated form, whereas that of haemocyanin from *Helix* is uninfluenced by ionisation is not at present clear. This may possibly be due to differences in the constitutions of the two proteins; one would feel inclined, however, to attribute the variation in affinity to the combined effects of aggregation and ionisation, were it not for the fact that Redfield, Coolidge and Hurd [1926] have found that haemocyanin from *Limulus* shows similar variations in affinity, although, as pointed out above, there are reasons for believing that it is molecularly dispersed in solution. It is possible that this discrepancy is due to the presence of inorganic salts, which might produce aggregation of the molecules of the protein, in the solutions examined by these authors. This possibility is supported by the fact that the oxygen dissociation curve of haemocyanin from *Limulus*, as determined by the same authors, is, in the presence of salts, of an S-shape; if the pigment were molecularly dispersed it should be of the hyperbolic type similar to that given by haemocyanin from *Helix*.

## SUMMARY.

The oxygen dissociation curve of haemocyanin from the snail (*Helix pomatia*) has been determined at a temperature of 23° and at  $p_H$  values ranging from 4.04 to 9.04. No detectable change in the curve with change in  $p_H$  was observed. Analysis of the curve indicates that it is of the hyperbolic type.

The following conclusions are drawn from this result. (1) The combination between haemocyanin from *Helix* and oxygen takes place according to the equation  $\text{Hcy} + \text{O}_2 = \text{HcyO}_2$ . (2) Each molecule of haemocyanin contains two atoms of copper. (3) The haemocyanin is molecularly dispersed in its solutions.

The bearing of these results on the different phenomena observed in the case of other haemocyanins is discussed.

The expenses of this investigation, which has been carried out during the tenure, by one of us, of a Carnegie Teaching Fellowship, have been met by grants from the Government Grant Committee of the Royal Society and the Moray Research Fund of this University.

## REFERENCES.

- Begemann (1924). Dissertation, Utrecht.  
Cohn (1925). *J. Biol. Chem.* **63**, xv.  
Hogben and Pinhey (1926). *Brit. J. Exp. Biol.* **4**, 203.  
Pantin and Hogben (1925). *J. Marine Biol. Assoc.* **13**, 970.  
Redfield, Coolidge and Hurd (1926). *J. Biol. Chem.* **69**, 475.  
Redfield, Coolidge and Montgomery (1928). *J. Biol. Chem.* **76**, 197.  
Redfield, Coolidge and Shotts (1928). *J. Biol. Chem.* **76**, 185.  
Stedman and Stedman (1925). *Biochem. J.* **19**, 544.  
—— — (1926, 1). *Biochem. J.* **20**, 938.  
—— — (1926, 2). *Biochem. J.* **20**, 949.  
—— — (1927). *Biochem. J.* **21**, 533.



# CXIII. THE ACTION OF CARBON MONOXIDE ON THE AUTOXIDATION OF SULPHYDRYL COMPOUNDS.

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*(Received June 28th, 1928.)*

THE work of Warburg [1928] and others has shown that the greater part of the oxygen uptake by living cells is due primarily to catalytic systems which are poisoned by small amounts of cyanide. According to Warburg's view of cell respiration the oxygen must react in the first place with some iron-containing cell catalyst which activates it, and he believes that the effect of cyanide is due to the formation of an inactive addition compound with this iron. It is well known that carbon monoxide also combines with many iron compounds, and Warburg [1927] has recently shown that cell respiration is also inhibited by carbon monoxide, and he has brought forward good evidence to show that its action is due, like that of the cyanide, to the inactivation of the catalytic iron compounds in the cell.

Warburg supposed that the cell respiration as a whole was poisoned by carbon monoxide, but his experimental data do not exclude the possibility that a part of the oxygen absorbed may be utilised by systems on which CO has no action. Such systems are in fact known to be present in tissues [Dixon, 1927].

Now it has been shown by Hopkins [1921] that part of the oxygen absorption by the tissues is due to the autoxidation of the —SH group of the glutathione present. This autoxidation is dependent on the presence of iron (or similar catalytic metals), and is prevented by the presence of small amounts of cyanide [Warburg and Sakuma, 1923; Harrison, 1924]; and it resembles in this respect the main part of the cell respiration.

In view of these facts it was thought to be desirable that a study of the effect of CO upon this autoxidation should be made, especially as in this system we have a case of a biologically important iron-catalysed oxidation which can be studied *in vitro* apart from other cell constituents. Moreover, by investigating the effect of carbon monoxide on the catalytic action of iron added to the system in various forms, one may expect to obtain information of value in interpreting results got with living tissues.

In the present paper experiments are given on the effect of CO on the catalysis by free iron and copper salts and by haematin of the autoxidation of cysteine and reduced glutathione.

The experiments were carried out by means of the Barcroft apparatus, using the technique already described [Dixon, 1927] for filling the apparatus with the gas mixtures. As light is known to diminish the inhibiting action of CO, the water-bath was equipped with a framework so arranged that a black cloth could be drawn over the whole in such a way as to exclude all light without interfering with the shaking or the reading of the manometers. Unless otherwise stated both flasks of each apparatus contained the same solutions, except that the sulphhydryl compound was present in the right-hand flasks only. The preparations of cysteine and reduced glutathione used, though pure specimens, were not free from traces of catalytic metals; but it was not necessary to use iron-free preparations in this work, since the procedure adopted in all the experiments was to compare the rates of oxygen uptake of identical

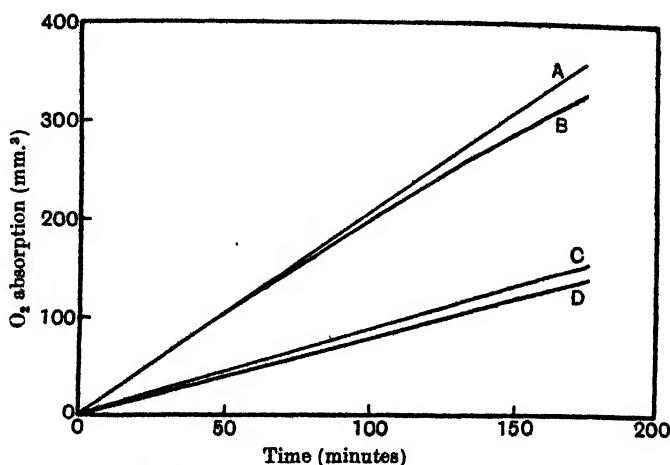


Fig. 1. Effect of CO on rate of autoxidation of cysteine. Curves A and B show the oxygen uptake of 20 mg. cysteine, curves C and D that of 10 mg. cysteine. In A and C the composition of the gas mixture was O<sub>2</sub> 5 %, CO 74 %, N<sub>2</sub> 21 %; in B and D O<sub>2</sub> 5 %, N<sub>2</sub> 95 %. Temperature 16°.

solutions in the presence and absence of carbon monoxide. The preparations also contained a small amount of their respective —SS— forms, owing to slight autoxidation having already taken place, and the total oxygen uptake was therefore rather below the theoretical; but this is of course quite immaterial. The experiments were carried out in all cases in phosphate buffer at  $p_H$  7.3, and the solutions of cysteine and glutathione were carefully adjusted to this  $p_H$  before use. The volume of liquid in each flask was always made up to 3 cc. with buffer solution.

Fig. 1 shows the autoxidation of cysteine in the presence and absence of CO, with two different concentrations of cysteine. No catalyst was added, and the autoxidation is due to the traces of catalytic metals already present in the preparation. It will be seen that the carbon monoxide produced no inhibition. Some similar results for glutathione are given in Fig. 4 (curves C and D).

Fig. 2 shows the effect of CO on the reaction when catalysed by iron salts. The complete curves are given, but the essential thing is of course the velocity during the linear part of the oxygen uptake. The concentration of the cysteine

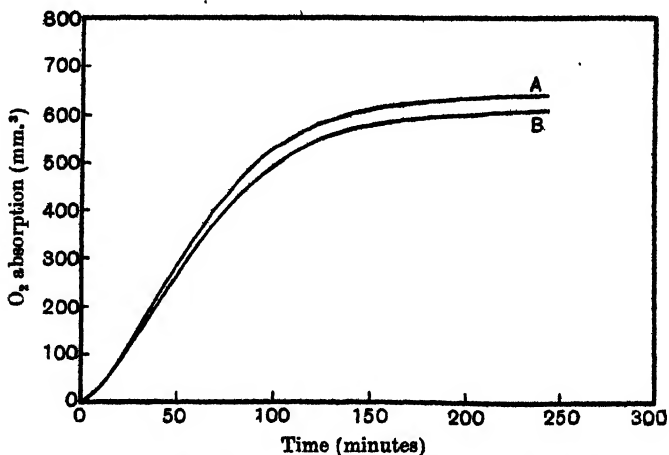


Fig. 2. *Effect of CO on catalysis of cysteine autoxidation by inorganic iron.* The solutions contained 20 mg. cysteine + 0.04 mg. Fe (added as  $\text{FeSO}_4$ ). Apparatus A contained O<sub>2</sub> 5%, CO 74%, N<sub>2</sub> 21%; apparatus B contained O<sub>2</sub> 5%, N<sub>2</sub> 95%. Temperature 17°.

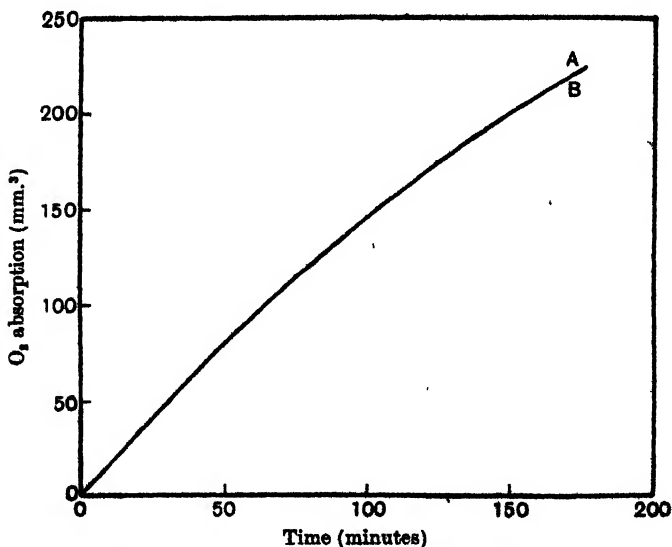


Fig. 3. *Effect of CO on catalysis of cysteine autoxidation by copper.* The solutions contained 8 mg. cysteine + a very small trace of copper sulphate. Gas mixtures as in Fig. 2. The curves in the presence and absence of CO coincided throughout. The experiment was carried out in duplicate (in different Barcroft apparatus), and the figure represents four completely coincident curves: two with, and two without CO. Temperature 18°.

was the same as in curves A and B of Fig. 1, but the addition of a small trace of ferrous sulphate had produced a threefold increase in the velocity of the oxidation. The curves show that although 70% of the oxygen uptake was

due to catalysis by the iron added, the carbon monoxide still produced no inhibition.

This result is interesting in view of the fact that Cremer [1928], in a paper which appeared while these experiments were being carried out, has shown that under such conditions the cysteine-iron complex combines at once with carbon monoxide to form an addition compound containing two molecules of CO for every atom of iron. These facts taken together show that the formation of a CO addition compound does not necessarily inactivate iron-containing catalysts, since, while Cremer's experiments make it certain that the iron is entirely or almost entirely converted into the addition compound, the curves show that there is no change in its catalytic activity.

The slight difference between the two curves, though not much greater than the experimental error, is probably due to the absorption of a small amount of CO in addition to the oxygen, since it corresponds roughly with the amount of CO which would be taken up to combine with the amount of iron present in forming Cremer's addition compound.

In Fig. 3 is shown a similar experiment in which copper was added instead of iron. Copper is a much more efficient catalyst than iron. Here only 0.1 cc. of an extremely dilute solution of copper sulphate was added, and it was found that under the conditions of this experiment the velocity of oxidation was thereby approximately doubled. At least half the oxygen uptake shown in Fig. 3 is therefore due to the catalytic action of copper, and the curves show that again carbon monoxide has no effect.

It was shown by Harrison [1924] that not only inorganic iron but also iron in the form of haematin could catalyse the autoxidation of sulphhydryl compounds, although the catalytic power of this form of iron was only about half that of iron salts. In order to investigate the effect of carbon monoxide upon this catalysis, solutions of haematin were prepared as follows. Pure crystalline haemin<sup>1</sup> was washed first with pure concentrated hydrochloric acid to remove any traces of free iron and then with water. Solutions containing 1 mg. per cc. were then made up by the careful addition of the minimum amount of dilute NaOH necessary to dissolve the haemin. It was found that the addition of these solutions in the amounts used in the experiments produced no detectable change in the  $p_H$  of the buffer solution used.

Some results showing the effect of CO on the catalysis by freshly dissolved haematin are given in Figs. 4 and 5. Fig. 4 shows experiments on glutathione, and Fig. 5, in curves *A* and *B*, gives results on cysteine. These curves are representative of a number of quite similar experiments.

In Fig. 4 the lower pair of curves gives the oxygen uptake by the glutathione alone in the presence and absence of CO; the upper pair gives the uptake in the presence of 0.5 mg. haematin, again in the presence and absence of CO. The difference between the upper and lower pairs of curves therefore represents

<sup>1</sup> I am indebted to Dr Kellin and also to Mr R. Hill for the specimens of haemin used in these experiments.

the oxygen uptake due to the catalytic action of the haematin. In this experiment the haematin was added to both flasks of each apparatus, and the sulphhydryl compound to the right-hand flasks only. In the experiments given

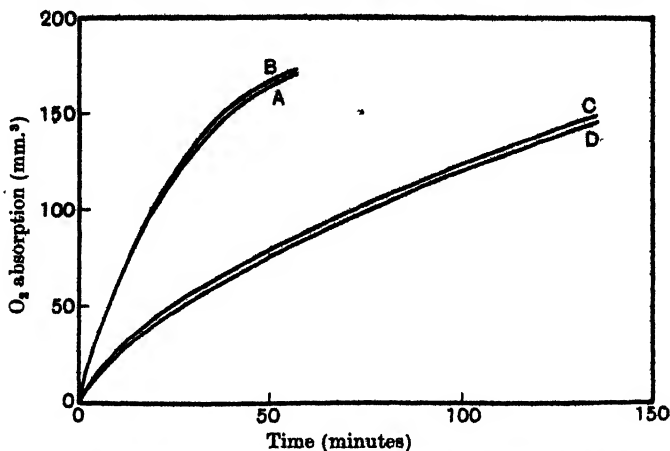


Fig. 4. Effect of CO on autoxidation of glutathione, alone and after the addition of haematin. Curves C and D give the oxygen uptake of 10 mg. glutathione; curves A and B of 10 mg. glutathione + 0.5 mg. haematin. A and C in the presence of CO, B and D in its absence. Gas mixtures as before. Temperature 18°. This experiment was also done in duplicate, and the duplicate curves all coincided completely, so that eight curves are represented in the figure.

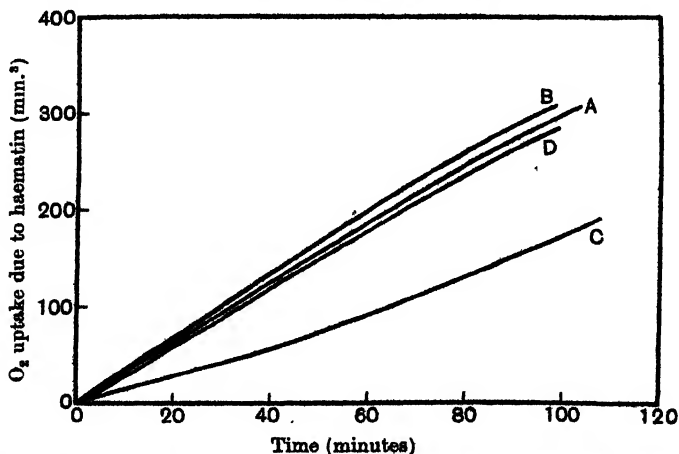


Fig. 5. Effect of CO on catalysis of cysteine autoxidation by freshly dissolved haematin and by haematin previously allowed to stand in solution. The curves give the oxygen uptake of 10 mg. cysteine + 0.5 mg. haematin, compensated for the spontaneous uptake of the cysteine alone. In curves A and B the haematin was freshly dissolved, in curves C and D the solution was several days old. A and C were in presence, and B and D in absence, of CO. Temperature 14°. In another experiment curve D lay between curves A and B.

in Fig. 5, however, the method was slightly modified. Here the haematin was added to the right-hand flasks only, and the cysteine was added to both flasks. By this means the oxygen uptake due to the catalytic metals already present in the cysteine was balanced out, and the reading of the manometers indicated

directly the oxygen uptake due to the haematin catalysis. This does not apply of course to the later phases of the experiment, when the rate of oxygen absorption in the right-hand flasks begins to fall off, but since we are only concerned with the rates of autoxidation during the first (linear) part of the curves this is no disadvantage. The figures may be taken as representing the actual oxygen uptake due to the haematin catalysis throughout the period covered by the curves given here.

These experiments make it quite clear that carbon monoxide produces no inhibition of the oxidation induced by haematin. They therefore stand in direct opposition to the recent results of Krebs [1928]<sup>1</sup>, who, using similar methods, found that CO produced a 70 % inhibition of the catalysis of the autoxidation of cysteine by haematin.

The discrepancy between my results and those of Krebs seems to be explained by the following observations. It is known that haematin in solution is unstable and breaks down fairly rapidly, giving a mixture of various less complex iron compounds. On allowing a solution of haematin to stand for several days it is usually found that a large part of the haematin has broken down. Such a solution is found to have precisely the same power to catalyse the oxygen uptake of cysteine as it had when freshly made up. That is to say, the mixture of iron compounds formed by the decomposition has exactly the same catalytic power as the original haematin. The behaviour towards CO is however very different. Whereas CO has no effect on the oxygen uptake induced by a freshly made up solution of haematin, it markedly inhibits that induced by the same solution after it has been allowed to stand for a few days. This is shown in Fig. 5, which is again representative of a number of experiments. Curves *A* and *B*, as already mentioned, represent the oxygen uptake due to freshly dissolved haematin in the presence and absence of CO. Curves *C* and *D* show the uptake produced by the same quantity of haematin which had been allowed to stand in solution for one week. Both haematin solutions were made up from the same sample of haemin, and it may be mentioned that the solution used for curves *C* and *D* was the identical solution which had been used, when freshly made up, for the experiment given in Fig. 4. In that experiment CO had no effect upon its catalytic action, whereas here a 50 % inhibition is produced.

It seems possible that Krebs may have used a stock solution of haematin, or haemin which had become partially decomposed, and that this may have been responsible for the inhibition he observed. At any rate, whether this is correct or not, I have never been able to detect any inhibition by CO when the haematin has been freshly made up, and have always found a marked inhibition after it has been allowed to stand for a few days before use; and this effect would seem to provide an adequate explanation of the discrepancy.

The catalytic action of the haematin decomposition products, like that of haematin itself, seems to be practically insensitive to the action of cyanide,

<sup>1</sup> This paper had not appeared at the time when these experiments were first carried out.

at any rate up to a concentration of  $M/300$  [see also Harrison, 1924]. It is clear then that cyanide and carbon monoxide are by no means always equivalent in their action as inhibitors of oxidation-catalysis by various forms of iron, since in the —SH system cyanide inhibits iron salts but not the haematin products, while carbon monoxide inhibits the haematin products but not iron salts. Other differences have been pointed out [Dixon, 1927]. These facts must be borne in mind when considering the interpretation of Warburg's results and the chemical nature of his iron-containing "Atmungsferment."

#### SUMMARY.

1. Carbon monoxide has no effect on the rate of the autoxidation of cysteine or reduced glutathione induced by the addition of iron or copper salts or by freshly dissolved haematin.

2. When a solution of haematin is allowed to stand for several days the haematin partly breaks down into a mixture of simpler iron compounds. This mixture has the same catalytic power as the original haematin, but the catalysis is now markedly inhibited by carbon monoxide.

I should like to express my sincere thanks to Sir F. G. Hopkins for his interest in this work and to Dr Keilin for his kindness in allowing me to use his apparatus and for supplying me with pure carbon monoxide.

#### REFERENCES.

- Cremer (1928). *Biochem. Z.* **194**, 221.  
Dixon (1927). *Biochem. J.* **21**, 1211.  
Harrison (1924). *Biochem. J.* **18**, 1009.  
Hopkins (1921). *Biochem. J.* **15**, 286.  
Krebs (1928). *Biochem. Z.* **193**, 347.  
Warburg (1927). *Biochem. Z.* **189**, 354.  
— (1928). *Über die katalytischen Wirkungen der lebendigen Substanz* (Berlin).  
Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.

## CXIV. THE ACTION OF ULTRA-VIOLET RAYS ON COMPLEMENT.

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THE normal complement system of serum depends for its action on the interaction or the presence in solution of several factors [cf. Browning, 1927], and this fact in all probability accounts for the varied and numerous methods which have been used to inactivate complement, since the destruction or inactivation of any one component is sufficient to reduce the complement activity of the serum to a very low and usually negligible value. To the three known factors which are concerned in complement action, a fourth component has been added [Gordon, Whitehead and Wormall, 1926, 1] and it has been found that a serum is only active in promoting haemolysis of sensitised cells when all four components are present in an active state. These four components differ widely in nature, having very different properties, and therefore the methods of inactivation are of a varied nature. Drastic treatment with acid or alkali usually destroys the whole activity, whilst, of the less drastic reagents, most appear to destroy the activity of the protein components. Inactivation by shaking [Jacoby and Schütze, 1910], or by the addition of acid in amounts greater than that required to bring the  $p_H$  to the isoelectric point of the proteins [Brooks, 1918], or by the action of pancreatic extracts [Michaelis and Skwirsky, 1910; Wormall, Whitehead and Gordon, 1925, 1927], appears to be effected by the destruction of the globulin and albumin components. A destruction of complement by ultra-violet rays was observed by Courmont, Nogier and Dufourt [1913] and later by other workers, but no explanation of this action appears to have been given. The temperature at which irradiation is carried out is of some significance [cf. Brooks, 1920, 1] but no appreciable part of the action can be attributed to temperature changes.

The investigations described here have been concerned with the study of the action of ultra-violet rays on complement as a whole and on the different components necessary for complement action. From such a study it is believed that information will be obtained about the general properties of the complement system and that the data will be useful in attempts to separate the different components. Many of the investigations in relation to the action of ultra-violet rays on biological fluids have been concerned with the effect on



the proteins present and in general it has been found that the most marked effects are due to changes in the state of the proteins; indeed, it is quite probable that, although the action of ultra-violet rays on biological fluids is of a complex and compound nature, the effects observed are due principally to changes in the state of the colloids. Thus it might be expected that any change in complement as a result of irradiation would be due to changes in the protein components and therefore that the results would be of some value in assessing the rôle of the serum-proteins in the complement system. Guinea-pig serum after irradiation for some time loses its power to haemolyse sensitised red cells, and by analysing the process of inactivation it has been found that the heat-labile protein components are mainly affected in this change. The relatively heat-stable components—the third component which can be removed from serum by yeast [von Dungern, 1900; Ehrlich and Sachs, 1902; Coca, 1914] or by zymin [Whitehead, Gordon and Wormall, 1925] and the fourth component which is inactivated by ammonia [Gordon, Whitehead and Wormall, 1926, 1]—are not altered appreciably during this period of irradiation. In addition it has been found that irradiation of the isolated mid-piece and end-piece fractions, which contain globulin plus third component and albumin plus fourth component respectively, gives similar results, the heat-labile component in each case being inactivated more rapidly than the relatively heat-stable components. Thus all these experiments tend to show that the protein factors only are sensitive to moderate irradiation with ultra-violet rays, and therefore the inactivation caused by irradiation runs parallel to some extent with that caused by heating the serum at 56° for 30 minutes: in each case the globulin and the albumin components are destroyed, but no significant change occurs in the third and fourth components which are associated with these protein factors respectively. Inactivation by ultra-violet rays does not appear to be due, however, to temperature changes in the serum since the changes are only observed when quartz vessels are used. The correlation between the action of ultra-violet rays and that of heat on complement is of interest in view of the results of Dreyer and Hanssen [1907] who found that albumin and globulin are readily coagulated by ultra-violet rays and that the phenomenon is a true coagulation, whilst more recently Spiegel-Adolf [1927] has found a relationship between the changes in serum-albumin on irradiation with ultra-violet rays and the process of heat coagulation. The results obtained by irradiation of guinea-pig serum with ultra-violet rays appear to furnish evidence in support of the view that some of the proteins of serum play an essential rôle in complement action. Besides the two protein components, termed the globulin and albumin components on account of their solubility and precipitation properties, two other factors are necessary for the full system. These two components, which are more resistant to heat and the action of ultra-violet rays than the globulin and albumin components, are not necessarily of protein nature, although the possibility cannot be excluded that they are more stable proteins which show a greater resistance to chemical and physico-chemical

treatment. This possibility appears unlikely, however, in view of the almost specific inactivation of the fourth component by small amounts of ammonia, and in view of many general differences between the relatively heat-labile and the relatively heat-stable components.

The general inactivation of complement by ultra-violet rays is markedly increased by previous dilution of the serum with 0.9 % NaCl, and a diluted serum (1 : 5) loses its specific haemolytic properties after a much shorter period of irradiation than that required to inactivate the undiluted serum. This greater inactivation in diluted solutions is probably due in part to an acceleration of the denaturation processes by a relative increase in the amount of sodium chloride, since Young [1922] observed a similar acceleration of the denaturation of serum-albumin by strong sunlight or the rays of an arc lamp when salts such as NaCl,  $(\text{NH}_4)_2\text{SO}_4$  and KCNS were added. Dilution of the serum with distilled water, however, increases the rate of inactivation by irradiation to an equivalent extent, and, although the partial precipitation of the globulin in such diluted solutions will facilitate the subsequent inactivation, it is probable that the concentration of the protein is the determining factor, and for this reason undiluted serum is most resistant to inactivation by ultra-violet rays or by heat.

Experiments have been carried out to determine whether inactivation by irradiation is due to oxidation changes, since Harris [1926] observed a marked uptake of oxygen by blood-plasma, ovalbumin, caseinogen and edestin on exposure to ultra-violet rays. Extensive inactivation of complement by irradiation occurs, however, in an atmosphere of hydrogen or *in vacuo*, although the velocity of inactivation appears to be somewhat less than that in the control experiments where the serum is exposed to the air. The primary inactivation, therefore, does not appear to be one of oxidation.

The kinetics of the destruction of complement by ultra-violet rays have been investigated fully by Brooks [1920, 1, 2], who reaches the conclusion that the photo-inactivation process is a monomolecular reaction, due to the removal of a substance responsible for the haemolytic action of the serum. No significant differences are observed when the rates of inactivation by heat of irradiated and non-irradiated sera are compared, nor does irradiation increase the susceptibility of the complement to inactivation by acids, and from this evidence Brooks considers that the haemolytic substance, which is inactivated by ultra-violet rays, is not a protein. No attempt was made, however, to reactivate the light-inactivated serum by the addition of separate fractions of complement and the evidence against the view that inactivation is effected through the protein components does not appear to be conclusive. Indeed, all our experiments on the irradiation of complement, some of which are described below, tend to show that the action of ultra-violet rays is similar to the action of heat, and that a change occurs in the state of the protein components which is not accompanied by any appreciable change in the relatively heat-stable components.

A series of experiments has also been carried out which showed that direct irradiation of guinea-pigs has no significant effect on the complement power of the serum, and that animals deprived of sunlight do not appear to be lacking in any way full complement activity. In view of these results it is of interest to note that Potthoff and Heuer [1922] observed very little change in antibody formation on exposure of animals to ultra-violet rays, and that Albela [1922] observed no modification of phagocytic action, as determined by opsonic action, after irradiating rabbits for long periods. The influence of prolonged continuous irradiation of guinea-pigs on the complement activity of the serum has been studied by Koopman [1924], who found that irradiation for 1 hour leads to a distinct rise in the complement activity of the serum, but this effect is only temporary, and 6 hours later the value has become normal. Irradiation of the animals for longer periods (2-3 hours) resulted in a marked diminution in the serum complement activity, and at death resulting from irradiation for 3 hours no complement was present in the serum. Since irradiation usually has a marked influence on the serum-calcium level and since the calcium of serum, probably the non-dialysable fraction, appears to be of importance in the full complement action [Gordon, Whitehead and Wormall, 1926, 2], determinations were made of the total serum-calcium with each guinea-pig used in the *in vivo* irradiation experiments described below. In the majority of cases the irradiated animals gave a slightly higher serum-calcium figure than the control, but small differences only were observed; in general, however, there appears to be some parallelism between the serum-calcium figures and the complement power. The amount of serum available, however, was not sufficient to allow determinations to be made of the amount of non-dialysable calcium in the sera, nor is it possible with the guinea-pig, which is the most suitable animal for complement work of this type, to record the changes in one animal over a period of weeks.

#### EXPERIMENTAL.

In all these experiments guinea-pig serum was used as the source of complement and the specific haemolytic properties were tested on a 4 % suspension of sensitised red cells. The total volume of solution was adjusted where necessary to 2.0 cc. by the addition of the requisite amount of 0.9 % NaCl, and the amount of haemolysis was recorded after incubation at 37° for  $\frac{1}{4}$  hour,  $\frac{1}{2}$  hour and 1 hour. Special precautions were taken to avoid excess of the serum containing the immune body (amboceptor), and immune sera with a high titre only were used; appreciable errors may be introduced by the use of excessive amounts of the immune serum for sensitisation, since this serum, although heated, contains the heat-stable factors necessary for complement action.

#### *Inactivation by ultra-violet rays of complement in undiluted and diluted sera.*

Serum, diluted and undiluted, was exposed to the rays from a Hanovia quartz mercury-vapour lamp, the solutions being contained in stoppered

Table I. *Effect of ultra-violet rays on undiluted and diluted sera contained in quartz tubes.*

Period of irradiation (hours)	Amount of undiluted serum used for complement test (cc.)	Undiluted serum. Haemolysis after incubation for		Serum diluted with equal volume of dist. water. Haemolysis after incubation for		Serum diluted with equal volume of 0.9 % NaCl. Haemolysis after incubation for		Serum diluted with 4 vols. dist. water. Haemolysis after incubation for		Serum diluted with 4 vols. 0.9 % NaCl. Haemolysis after incubation for	
		$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour
0	-	+	+	+	+	+	+	+	+	+	+
	0.10	+	+	+	+	+	+	+	+	+	+
	0.06	+	+	+	+	+	+	+	+	+	+
	0.04	-	+	+	+	-	+	+	+	+	+
	0.02	-	+	+	+	+	+	-	+	+	+
1	0.01	-	+	+	+	-	+	-	+	-	+
	0.10	+	+	+	+	+	+	-	+	-	+
	0.06	-	+	+	+	-	+	-	+	-	+
	0.04	-	+	+	+	-	+	-	+	-	+
	0.02	-	+	+	+	-	+	-	+	-	+
2	0.01	-	+	+	+	-	+	-	+	-	+
	0.10	+	+	+	+	-	+	-	+	-	+
	0.06	-	+	+	+	-	+	-	+	-	+
	0.04	-	+	+	+	-	+	-	+	-	+
	0.02	-	+	+	+	-	+	-	+	-	+
3	0.01	-	+	+	+	-	+	-	+	-	+
	0.10	-	+	+	+	-	+	-	+	-	+
	0.06	-	+	+	+	-	+	-	+	-	+
	0.04	-	+	+	+	-	+	-	+	-	+
	0.02	-	+	+	+	-	+	-	+	-	+

quartz tubes held about 9 inches from the lamp. Samples were withdrawn at intervals from these solutions and from "control" solutions contained in glass tubes, and all the samples tested for complement power. The determination of complement power was made by pipetting accurately three different amounts of each solution, diluting these samples with 0.9 % NaCl and setting up dilution ranges with each sample. The samples from the serum diluted with distilled water were treated with the necessary amount of 10 % NaCl to give 0.9 % NaCl in the final solution. Table I gives a typical result, the actual speed of inactivation varying somewhat with the serum used and with the distance of the serum from the lamp. The amounts of serum used are recorded in the table in cc. of original or undiluted serum.

From these experiments (Table I) it is found that complement is readily inactivated by ultra-violet rays, and that serum diluted with water or 0.9 % NaCl loses its complement activity much more quickly under irradiation than does undiluted serum. The rate of inactivation of complement contained in glass tubes by ultra-violet rays under similar conditions is very slow and almost negligible (Table II), and, whereas the serum contained in quartz tubes loses a large part of its complement activity in 1 hour and almost the whole of its complement in 2 hours, the serum contained in a glass tube retains almost the whole of its complement activity after irradiation for 3 hours. The small amount of inactivation which does occur may be attributed to the passage of a small percentage of the active rays through the glass.

Table II. *Complement activity of serum contained in glass tubes and irradiated with ultra-violet rays.*

Amount of undiluted serum used	Incubation for							
	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour	$\frac{1}{2}$ hour	1 hour
0.10 cc.	++++	++++	+++	++++	+++	++++	+++	++++
0.06 cc.	++	++++	++	++++	+	++++	+	++++
0.04 cc.	-	++++	-	++++	-	++	-	++
0.02 cc.	-	++	-	++	-	+	-	-
0.01 cc.	-	-	-	-	-	-	-	-
Duration of irradiation (hours)	0		1		2		3	

*The action of ultra-violet rays on the various components of complement.*

Serum was exposed to ultra-violet rays until all the complement activity was lost and the inactivated serum tested for the relatively heat-stable components; (a) for the "third component" by determining the ability to reactivate zymine-inactivated serum, and (b) for the fourth component by determining the power to reactivate ammonia-inactivated serum. It was found (Table III) that an irradiated serum which had lost all detectable complement activity still contained these heat-stable components. In a similar experiment it was found that serum heated at 56° for  $\frac{1}{2}$  hour and containing therefore the heat-stable but not the heat-labile components of complement, does not lose these heat-

stable components to any appreciable extent when exposed to ultra-violet rays under conditions which would cause rapid inactivation of the heat-labile components.

Table III. *Effect of ultra-violet rays on the various components of complement.*

Amount of undiluted serum used	Duration of irradiation	Haemolysis after incubation for 1 hour		
		+0.9 % NaCl only	+0.05 cc. NH <sub>3</sub> -inactivated serum	+0.05 cc. zymine-inactivated serum
Unheated serum:				
0.10 cc.	0	++++	++++	++++
0.05 cc.	0	++++	++++	++++
0.02 cc.	0	++	+++	+++
0.10 cc.	1	++++	++++	++++
0.05 cc.	1	++	++++	++++
0.02 cc.	1	—	++	++
0.10 cc.	2	++	++++	++++
0.05 cc.	2	—	++++	++++
0.02 cc.	2	—	+	+
0.10 cc.	3	—	++++	++++
0.05 cc.	3	—	+++	+++
0.02 cc.	3	—	+	—
Serum heated at 56° for 30 minutes:				
0.10 cc.	0	—	++++	++++
0.05 cc.	0	—	++++	++++
0.02 cc.	0	—	++	++
0.10 cc.	2	—	++++	++++
0.05 cc.	2	—	++++	++++
0.02 cc.	2	—	+	—
0.10 cc.	3	—	++++	++++
0.05 cc.	3	—	+++	+++
0.02 cc.	3	—	+	—

The  $\text{NH}_3$ -treated serum and the zymine-treated serum had no complement power but could be activated by heated "end-piece" and heated "mid-piece" respectively.

Confirmatory evidence of the greater sensibility of the proteins or heat-labile components to the action of ultra-violet rays was obtained by irradiating the isolated "mid-piece" (or globulin) and "end-piece" (or albumin) fractions of complement. These fractions were obtained by the  $\text{CO}_2$  method of Liefmann [1909], the excess of  $\text{CO}_2$  being removed by evacuation. Both fractions were adjusted to  $p_{\text{H}}$  7.5 and the separated fractions had no haemolytic power but together possessed almost the full complement activity of the original serum. The results, which are typical of many experiments, are recorded in Table IV and they show that the isolated fractions are much more sensitive to irradiation than the original serum, possibly on account of the dilution. The results are similar to those with undiluted serum in that they show that the relatively heat-stable components are more resistant to destruction by ultra-violet rays than the heat-labile components. It is interesting to note that the third component is less resistant to irradiation than the fourth component.

Table IV. *Irradiation of the isolated "mid-piece" and "end-piece" fractions of serum.*

Solution irradiated	Duration of irradiation (mins.)	Haemolysis after incubation for 1 hour		
		+0.5 cc. end-piece (1 : 10)	+0.5 cc. NH <sub>4</sub> -treated serum (1 : 10)	+0.5 cc. symin-treated serum (1 : 10)
0.5 cc. mid-piece (1 : 10)	0	++++	—	++++
0.5 cc. "	15	—	—	++ or +++
0.5 cc. "	30	—	—	—
0.5 cc. "	45	—	—	—
		+0.5 cc. mid-piece (1 : 10)		
0.5 cc. end-piece (1 : 10)	0	++++	++++	+ or —
0.5 cc. "	15	—	++	—
0.5 cc. "	30	—	++	—
0.5 cc. "	45	—	—	—

*Influence of oxygen on the inactivation of complement by ultra-violet rays.*

Guinea-pig serum was exposed to a vacuum to remove as much dissolved oxygen as possible and divided into two portions, each fraction being placed in a "vitaglass" Thunberg tube. One tube was evacuated and the other kept open to the air and both tubes were exposed to ultra-violet rays at a distance of about 9 inches from the lamp. Samples were withdrawn at intervals and the complement activities of the two sera were compared. The differences observed were very small, but all experiments showed that a slightly greater loss of complement activity occurred when the irradiated serum was exposed to the air. Similar differences were observed when the Thunberg tubes were reversed, to obviate any error due to differences in permeability of the walls of the tubes to ultra-violet rays, or when stoppered quartz tubes were used; the velocity of inactivation when quartz tubes were used was much greater than those indicated by Table V, which gives typical results for the experiments with "vitaglass" Thunberg tubes. Experiments with serum diluted with 0.9 % NaCl gave similar results with a much greater velocity of inactivation.

Table V. *Irradiation of guinea-pig serum in presence and absence of air.*

Serum in "vitaglass" tubes exposed to:	Amount of serum used for complement test	Duration of irradiation (hours)			
		0	1	2	3
air	0.10 cc.	++++	++++	++++	++
	0.05 cc.	++++	++++	++	+
	0.02 cc.	+++	++	+	—
vacuum	0.10 cc.	++++	++++	++++	++++
	0.05 cc.	++++	++++	+++	++
	0.02 cc.	+++	++	++	—

*Influence of irradiation of guinea-pigs on the complement activity of the serum.*

Experiments have been carried out to determine what effect, if any, is produced on the complement activity of the serum of guinea-pigs when these

animals are subjected to irradiation at definite intervals. Two dozen large guinea-pigs were kept in the dark and fed on a standard diet of bran, oats, hay and turnips; one-half of the pigs were exposed to the rays of the mercury vapour lamp (Hanovia type) three times a week, the distance from the lamp being 18 or 20 inches and the duration of the irradiation 10 minutes during the first 6 weeks and 15 minutes afterwards. One irradiated guinea-pig and the corresponding control non-irradiated animal of the same size, age and colour were killed each week and the complement activity and the calcium content of the sera were determined. The calcium determinations were made

Table VI. *Complement powers of the sera of irradiated and non-irradiated guinea-pigs.*

No. of weeks		No. of irradiations	Amount of undiluted serum used (cc.)					Calcium content of serum (mg. per 100 cc.)
			0.100, 0.075, 0.050, 0.037	0.025	0.018	0.012	0.006	
1	Irradiated	3	++++	++++	++++	++++	++	—
	Non-irradiated	3	++++	++++	++++	++++	+	—
2	Irradiated	6	++++	++++	++++	++++	+++	13.4
	Non-irradiated	6	++++	+++	++	+	—	12.3
3	Irradiated	9	++++	++++	++	+	—	12.7
	Non-irradiated	9	++++	++++	++++	++++	—	13.3
4	Irradiated	12	++++	++++	++++	++++	+	13.5
	Non-irradiated	12	++++	++++	+++	+	—	13.0
5	Irradiated	15	++++	++++	++++	+++	—	12.5
	Non-irradiated	15	++++	++++	++	—	—	11.4
6	Irradiated	18	++++	+++	++	++	—	9.3
	Non-irradiated	18	++++	+++	++	+	—	9.5
7	Irradiated	21	++++	+++	+++	++	—	10.0
	Non-irradiated	21	++++	++++	++++	+++	—	10.6
8	Irradiated	24	++++	++++	+++	++	—	12.8
	Non-irradiated	24	++++	++++	++	++	—	11.2
9	Irradiated	27	++++	++++	++++	++	—	12.5
	Non-irradiated	27	++++	+++	++	+	—	11.4
10	Irradiated	30	++++	++++	+++	++	—	10.5
	Non-irradiated	30	++++	++++	++++	+++	—	10.5
11	Irradiated	33	++++	++++	++++	+++	+	13.3
	Non-irradiated	33	++++	++++	+++	++	—	12.4

by the modification by Clark and Collip [1925] of the method of Kramer and Tisdall [1921]. To compare the complement powers of the two sera, varying amounts of the sera were diluted accurately with 0.9 % NaCl and the haemolytic powers of these diluted solutions for sensitised cells measured. No strict comparison can be drawn, however, between the results obtained on any one occasion and those obtained earlier or later in the series, since it does not appear possible to obtain accurate standardisation of the system red cells plus immune serum. Standardisation of this system would be necessary for an absolute determination of complement activity since it has been shown by several authors that complement and sensitiser are interdependent [Morgenroth and Sachs, 1902; Noguchi, 1923; Hyde and Parsons, 1927] and relatively less complement is required to effect the complete haemolysis of red cells when excess of immune body is present. In our experiments we have observed



a similar relationship and in addition it has been found necessary to reduce the amount of immune serum used for sensitisation of the red cells to a minimum to avoid adding relatively heat-stable components of complement. When the same suspension of sensitised cells is used, however, and if the amount of haemolysis is recorded after varying intervals of time (e.g.  $\frac{1}{4}$  hour,  $\frac{1}{2}$  hour and 1 hour), a comparison of the complement activities of two or more sera can be made. In Table VI the results given are those recorded after incubation for  $\frac{1}{2}$  hour, the results for the other periods showing a similar relationship, whilst the results with amounts of serum intermediate between those recorded are not given. The control animal for one guinea-pig died and the results are recorded for 11 sets of guinea-pigs only. The results indicate that irradiation of guinea-pigs with ultra-violet rays has very little, if any, influence on the complement activity of the serum, and that guinea-pigs kept in the dark as far as possible do not show any marked loss in serum complement activity within the limits of this experiment. The calcium contents of the sera also show no marked differences, but it is of interest to note that where differences in the complement activity do occur between the irradiated and the control animals, there is also a slight difference in the calcium values and the two appear to run parallel.

#### SUMMARY.

1. The destructive action of ultra-violet rays on the complement of guinea-pig serum is primarily due to inactivation or destruction of the heat-labile protein components. More intensive irradiation leads to a slight loss in the relatively heat-stable components, but the rate of this inactivation is very small with undiluted serum.
2. The relatively heat-stable components present in guinea-pig serum, which has been heated at  $56^{\circ}$  for 30 minutes, are not affected to any appreciable extent by irradiation sufficient to destroy the complete complement activity of the unheated serum.
3. Irradiation of the isolated globulin and albumin fractions of complement leads to similar conclusions, but the inactivation is much more rapid with these dilute solutions.
4. The rate of inactivation of complement by ultra-violet rays is increased greatly by dilution of the serum with distilled water or 0.9 % NaCl.
5. The inactivation of complement by ultra-violet rays does not appear to be due to oxidative effects, but the rate of inactivation *in vacuo* is slightly less than that in air.
6. Irradiation of guinea-pigs with ultra-violet rays does not increase appreciably the complement activity of the serum, nor does the serum of guinea-pigs kept in the dark show any marked loss in this power.

The authors desire to express their indebtedness to the Medical Research Council for part-time personal grants and for grants which have partly defrayed the expenses of this research.

## REFERENCES.

- Albela (1922). *Deutsch. med. Woch.* **48**, 1347.  
Brooks (1918). *J. Med. Res.* **38**, 345.  
—— (1920, 1). *J. Gen. Physiol.* **3**, 169.  
—— (1920, 2). *J. Gen. Physiol.* **3**, 185.  
Browning (1927). *Brit. Med. J.* **ii**, 978.  
Clark and Collip (1925). *J. Biol. Chem.* **63**, 461.  
Coca (1914). *Z. Immunitätsforsch.* **21**, 604.  
Courmont, Nogier and Dufourt (1913). *Compt. Rend. Soc. Biol.* **74**, 1152.  
Dreyer and Hanssen (1907). *Compt. Rend. Acad. Sci.* **145**, 234.  
von Dungern (1900). *Munch. med. Woch.* **47**, 677.  
Ehrlich and Sachs (1902). *Berl. klin. Woch.* **39**, 297, 335 and 492.  
Gordon, Whitehead and Wormall (1926, 1). *Biochem. J.* **20**, 1028.  
—— ——— (1926, 2). *Biochem. J.* **20**, 1036.  
Harris (1926). *Biochem. J.* **20**, 271, 280 and 288.  
Hyde and Parsons (1927). *Amer. J. Hyg.* **7**, 11.  
Jacoby and Schütze (1910). *Z. Immunitätsforsch.* (Orig.), **4**, 730.  
Koopman (1924). *Deutsch. med. Woch.* **50**, 277.  
Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.  
Liefmann (1909). *Munch. med. Woch.* **56**, 2097.  
Michaelis and Skwirsky (1910). *Z. Immunitätsforsch.* (Orig.), **7**, 497.  
Morgenroth and Sachs (1902). *Berlin klin. Woch.* **35**, 817.  
Noguchi (1923). Cited from Hyde and Parsons.  
Potthoff and Heuer (1922). *Zentr. Bakt. Par.* (Orig.), **88**, 299.  
Spiegel-Adolf (1927). *Biochem. Z.* **186**, 181.  
Whitehead, Gordon and Wormall (1925). *Biochem. J.* **19**, 618.  
Wormall, Whitehead and Gordon (1925). *J. Immunol.* **10**, 587.  
—— ——— (1927). *J. Immunol.* **13**, 451.  
Young (1922). *Proc. Roy. Soc. Lond. B*, **93**, 235.

# CXV. THE CREATINE-CREATININE EQUILIBRIUM. THE APPARENT DISSOCIATION CONSTANTS OF CREATINE AND CREATININE.

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*(Received June 30th, 1928.)*

THE facile conversion of creatine into creatinine under the influence of strong acids and the partial reversal of the reaction in neutral and in alkaline solutions have long been familiar. Yet only recently has there become available any quantitative data upon the equilibrium conditions. Hahn and Barkan [1920] were the first to report any systematic kinetic studies. They determined the equilibrium constant in solutions of sodium hydroxide of varying concentration, observed that the component velocities increased with increasing  $[\text{OH}^-]$  and showed that the order of the reaction creatinine  $\rightarrow$  creatine, under these conditions, was that of a reversible monomolecular system. In a molar solution of hydrochloric acid, on the other hand, the reverse reaction went to completion and followed the course of a simple monomolecular change. Hahn and Meyer [1923] later reported a few observations which indicated that the velocity of this reaction in buffered solutions increased rapidly from  $p_{\text{H}}$  6 to 4. A more elaborate study of this system has been made by Edgar and his associates. Edgar and Wakefield [1923] determined the monomolecular velocity constants ( $k_2$ ) of the dehydration of creatine in hydrochloric acid solutions of varying concentration and at various temperatures. They succeeded in relating  $k_2$  to the temperature by means of the Arrhenius equation and, further, concluded that  $k_2$  was, probably, proportional to the hydrogen ion activity. Finally, Edgar and Shiver [1925] have made an extensive series of determinations of the equilibrium constant ( $K$ ) at  $50^\circ$  in buffered solutions of  $p_{\text{H}}$  values 1 to 6. Hahn and Barkan had suggested that their observations could be interpreted upon the assumption that the molecular species whose concentrations determined the equilibrium were the undissociated molecules of creatine and of creatinine. Confirming this, Edgar and Shiver obtained fairly satisfactory agreement between the observed values of  $K$  and those calculated from the dissociation constants of the two bases and the value of  $K$  in unbuffered solution (i.e. where the two reactants were not significantly dissociated).

Consideration of the results summarised above will indicate that, although the hypothesis of Hahn and Barkan has been useful in co-ordinating the equilibrium data, it fails to comprehend the relations between  $[H^+]$  and the velocities of the reactions. If the equilibrium be determined by the ratio of the concentrations of the undissociated molecules of creatine and creatinine, the velocities of the two contributing reactions should be governed by the same factors. Thus, if  $k_b$  be the dissociation constant of either reactant and  $k$  the velocity constant for its decomposition into the other, then  $k$  should vary with  $\frac{[OH^-]}{k_b + [OH^-]}$ . That is to say, the velocity should be inversely proportional to  $[H^+]$  on the acid side of the buffer range of  $k_b$  and should be independent of  $[H^+]$  on the alkaline side. The available experimental evidence, however, indicates that the velocities are proportional to  $[H^+]$  in solutions of strong acids and are inversely proportional in strongly alkaline solution. The reactions under discussion are of such direct biological interest that it was decided to undertake a series of kinetic studies in buffered solutions between  $p_H$  1 and 10 as an attempt to elucidate these discrepancies.

It was necessary, in the first place, that there should be available dependable values for the dissociation constants of creatine and creatinine. Since the values in the literature differ rather seriously, a redetermination of these constants was undertaken.

#### DISSOCIATION CONSTANTS OF CREATINE AND CREATININE.

The method employed was that of electrometric titration of dilute solutions of the two bases with standard hydrochloric acid in the presence of the hydrogen electrode. The routine technique of this laboratory has already been described [Cannan and Knight, 1927]. The reference electrode was a saturated calomel cell which was standardised against 0.05 *M* acid potassium phthalate [Clark, 1922]. Two palladinised gold-plated platinum electrodes were employed as duplicate hydrogen electrodes. No difficulty was encountered in attaining stable potentials in any of the solutions titrated and the two electrodes agreed within 0.3 mv. at all significant points on the titration curves.

The creatine was prepared from a good commercial sample by repeated recrystallisation from water. After drying to constant weight over calcium chloride, a typical preparation gave

Nitrogen (Kjeldahl) ...	28.19 %	Water 12.18 %
Theory for $C_4H_9O_2N_3 \cdot H_2O$	28.19	12.08

A saturated solution gave no reaction for creatinine upon applying Weyl's test.

Creatinine was prepared from the creatine by treating the latter with hydrochloric acid gas and subsequent liberation of the base by aqueous ammonia. The product was recrystallised from acetone [Edgar and Hinegardner, 1928]. Nitrogen and water determinations were quantitative for anhydrous creatinine. Folin's colorimetric method for the determination of

creatinine (using creatinine picrate as standard) gave results in agreement with the nitrogen values, but this method is, admittedly, not sufficiently accurate to detect traces of impurity in creatinine.

It will be convenient, throughout the paper, to conduct the discussion in terms of hydrogen ions rather than of hydroxyl ions and, consequently, all constants will be treated as though they were acid constants. That is to say, the kation of a base will be regarded as an acid which dissociates a hydrogen ion [Bronsted, 1923]. The constants so derived ( $k'$ ) are related to the familiar  $k_b$  values by the equation  $p_{k'} = p_{k_b} - p_{k_b}$ .

Table I. *Uncorrected apparent dissociation constant of creatinine.*

Authors	Molar conc.	Temp.	$p_{k'}$	$k_b$
Wood [1903]	0.1	40.2	2.97	$3.57 \times 10^{-11}$
McNally [1926]	—	40.0	4.42	$1.01 \times 10^{-9}$
Cannan and Shore	0.1	30.0	4.77	$0.98 \times 10^{-9}$
"	0.02	30.0	4.72	$0.15 \times 10^{-9}$
"	0.1	25.0	4.78	$0.76 \times 10^{-9}$
McNally [1926]	—	25.0	4.71	$0.70 \times 10^{-9}$
Eadie and Hunter [1926]	0.1	20.0	4.87	$0.64 \times 10^{-9}$
Hahn and Barkan [1920]	0.04	17.0	4.44	$0.19 \times 10^{-9}$
Cannan and Shore	0.02	15.0	4.91	$0.47 \times 10^{-9}$

Wood, and Hahn and Barkan calculated  $k_b$  from the degree of hydrolysis of solutions of the hydrochloride; Eadie and Hunter employed the electrometric titration; McNally's results are the mean of results from the conductance, hydrogen ion concentration and distribution of the hydrochloride.

In Table I are assembled several determinations of  $p_{k'}$  for creatinine together with values calculated from the  $k_b$  values recorded in the literature. The important effect of temperature upon the constant is evident and renders difficult the comparison of the results of different observers. But it would seem that, apart from the two earliest determinations which were made with methods open to considerable experimental errors, the various values are in substantial agreement. It is unnecessary, therefore, to report our experimental data in any greater detail. For purposes of the analysis of the kinetic studies which follow, the value for the dissociation constant of creatinine at 30° will be taken to be  $k' = 1.90 \times 10^{-5}$ , *i.e.*  $p_{k'} = 4.72$ .

The case of creatine is less satisfactory. The various determinations are summarised in Table II. In Table III is given the analysis of a typical titration curve to indicate the degree of concordance of the data. The calculations have been made with the aid of the Henderson-Hasselbalch equation. The values of  $[H^+]$ , used in calculating the "corrected equivalents of acid," are obtained from the observed  $p_H$  after correction for the activity of the hydrogen ion by the equation  $\log \tau_H = 0.20 \sqrt{\Sigma iv^2}$  [Simms, 1926].  $\tau_H$  is the activity coefficient ratio for the hydrogen ion,  $\Sigma iv^2$  is the sum of all the ion concentrations each multiplied by the  $z$  power of its valency. The value of  $z$  was assumed to be unity. The constants have not been corrected for activity.

Table II. *Uncorrected apparent dissociation constants of creatine.*

Authors	Molar conc.	Temp.	$k'_1$	$p_{K'_1}$
Wood [1903]	0.1	40.2	$2.1 \times 10^{-3}$	2.68
Cannan and Shore	0.1	30.0	$2.4 \times 10^{-3}$	2.62
"	0.02	30.0	$2.4 \times 10^{-3}$	2.62
"	0.1	25.0	$2.2 \times 10^{-3}$	2.66
Eadie and Hunter [1926]	0.05	20.0	$0.9 \times 10^{-3}$	3.05
Hahn and Barkan [1920]	0.04	17.0	$1.4 \times 10^{-3}$	2.85
Cannan and Shore	0.05	17.0	$2.45 \times 10^{-3}$	2.61

Table III. *Titration of 50 cc. 0.02 M creatine with 0.1 M hydrochloric acid.*

Titre	$p_H$	[H <sup>+</sup> ] corrected	Corrected equiv. acid - [HCl] - [H <sup>+</sup> ] [creatine]	$\log \frac{a}{1-a}$	$p_{K'_1}$
0.00	5.77	—	0.00	—	—
0.20	4.41	$39.0 \times 10^{-6}$	0.0181	-1.74	2.67
0.52	3.95	$11.2 \times 10^{-6}$	0.0452	1.33	2.62
1.02	3.64	$23.4 \times 10^{-6}$	0.0901	1.01	2.63
2.01	3.30	$51.3 \times 10^{-6}$	0.1744	0.68	2.62
3.02	3.08	$85.1 \times 10^{-6}$	0.2568	0.47	2.63
4.00	2.92	$123.0 \times 10^{-6}$	0.3337	0.30	2.62
4.98	2.79	$166.0 \times 10^{-6}$	0.4064	0.16	2.63
6.00	2.67	$224.0 \times 10^{-6}$	0.4749	-0.04	2.63
8.02	2.48	$347.0 \times 10^{-6}$	0.6010	+0.18	2.64
10.00	2.34	$501.0 \times 10^{-6}$	0.7000	0.36	2.70
13.03	2.15	$794.0 \times 10^{-6}$	0.8016	0.61	2.76

It is probable that the last two calculations suffer by reason of the uncertainty of the correction for hydrogen ion activity.

It will be seen from Table II that differences exist between the determinations of different observers which cannot be attributed to differences of temperature or of concentration. In particular, it is difficult to explain the conflicting results of Eadie and Hunter and of ourselves since the same method was employed and was prosecuted with the same degree of precision. No plausible source of error in the titrimetric method peculiar to creatine suggests itself. The possibility of a significant amount of conversion of creatine into creatinine during the course of a titration seems to be excluded by the velocity measurements recorded in the second part of this paper. Provisionally we will take the value for  $k'$  at 30° as  $2.40 \times 10^{-3}$ , i.e.  $p_K = 2.62$ .

A question of some interest to the chemical behaviour of creatine arises from a consideration of its electrolytic dissociation. The conventional formula for creatine contains both a carboxyl and an amino-group. Creatine might be expected to behave, therefore, as an ampholyte. Only basic properties are, however, evident in its chemical behaviour and only one dissociation constant is detected by titration. This is, therefore, described as a basic constant. Hahn and Fasold [1925] have, however, found that the solubility of creatine in solutions of sodium hydroxide is greater than in water and they conclude that some dissociation of creatine as an acid occurs in solutions of great hydroxyl concentration. From their observations they calculate a value of 14.28 for  $p_{K_a}$ . Now the allocation of the first dissociation constant of

creatinine to the amino-group and the assignment of only negligible acid properties to the carboxyl is difficult to justify upon the grounds of organic chemical experience. Yet it is usual to describe creatine as a base. A more plausible interpretation of the acid-base behaviour of this substance would seem to follow the application to it of Bjerrum's [1923] treatment of the amino-acids. The first constant ( $k_1' = k_w/k_b$ ) then becomes the acidic constant and the second—in this case, inaccessible—constant ( $k_2'$ ) is the association constant of the basic group. With this assignment of constants creatine becomes an acid comparable in strength with other carboxylic acids. At the same time a new difficulty is created for it is required that the basic dissociation shall be as great as that of the alkali hydroxides. It would be difficult to concede this to a simple amino-group and it is of interest, therefore, that creatine does not behave as a primary amine either towards nitrous acid or towards formaldehyde. In this connection the strong basic properties and anomalous behaviour of guanidine itself will be recalled. It is significant that several of the structural formulae which have been proposed to explain the anomalous reaction with nitrous acid contain a nitrogenous group which might be expected to dissociate strongly as a base [Hunter, 1928, p. 99].

The above considerations in no way prejudice the application of the dissociation constants to the co-ordination of kinetic data. It is a matter of no immediate moment whether the velocity of dehydration of creatine is determined by the concentration of undissociated creatine or of "zwitterion"—the mathematical relation to  $k_1'$  remains unmodified.

#### CREATINE-CREATININE EQUILIBRIUM.

Solutions of creatine (0.0106 *M*) and of creatinine (0.00354 *M*) were prepared in a series of the 0.05 *M* buffers recommended by Clark [1922]. The mixtures were covered with 10 cc. of toluene and stored in stoppered bottles in an air-bath maintained at  $30^\circ \pm 1^\circ$ . At intervals appropriate to each experiment, a sample was removed and the concentration of creatinine present was determined by the method of Folin. The standard solutions for this method were prepared from a purified specimen of creatinine picrate. The  $p_H$  values of the various reaction mixtures were determined at the beginning, and again at the conclusion, of each experiment by means of the hydrogen electrode. At the end of each experiment determination was also made of the total creatine + creatinine. In agreement with other investigators it was found that some conversion occurred of these two substances into products which reacted neither as creatine nor as creatinine. The extent of the loss during the period of experiment varied from 0.5 to 5 % according to the  $p_H$  of the solution. In view of the temperature at which the solutions were maintained and of the precarious antiseptic properties of toluene over long periods, the occurrence of bacterial decomposition may be suspected. This source of error cannot be absolutely excluded but the results are so concordant amongst themselves and fit in so well with the equilibrium data of Edgar and Shiver

(obtained at temperatures from 25 to 100°) and the relation of the losses to  $p_H$  is such that we are persuaded that the observed destruction of reactants was not due to bacteria but to irreversible chemical decomposition to an extent similar to that recorded by the earlier observers. In any case these irreversible changes are not sufficient to explain the gross relations between the velocities and  $p_H$  which will be established.

Fig. 1 presents a summary of one series of observations upon the two reactions. It presents several unexpected relations of the velocities to  $p_H$ —notably the well-defined  $p_H$  optima.

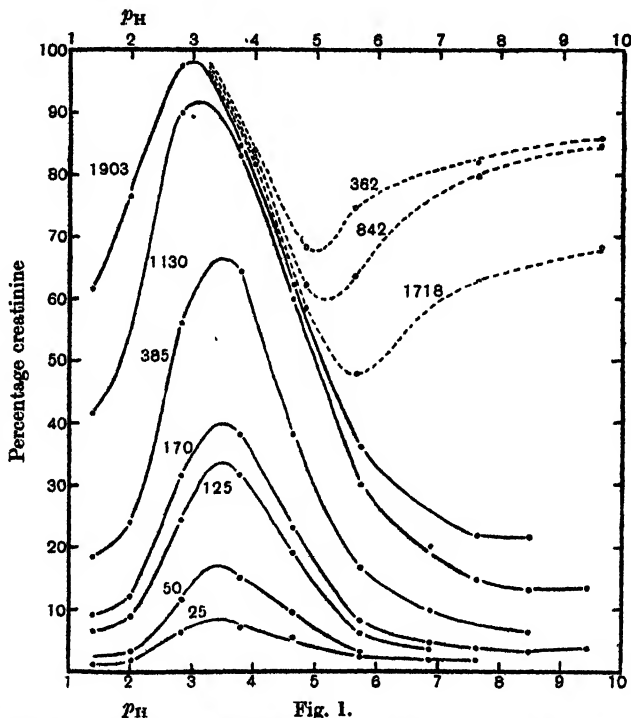


Fig. 1.

- Percentage creatinine formed in solutions of creatine at times (hours) indicated.
- Percentage creatinine remaining in solutions of creatine.

The data upon the change creatine→creatinine are more extensive than those for the reverse reaction and the analysis of the former will suffice to bring out all the important relations. The velocity constants for the latter reaction, in the  $p_H$  range where it is significant, fully confirm these relations.

In solutions acid to about  $p_H$  3 the conversion of creatine into creatinine is seen to be substantially irreversible. In such solutions the reaction would be expected, therefore, to proceed as one of the first order. The first half of Table IV indicates the measure of constancy of the monomolecular velocity constants ( $k_1$ ) derived from a typical experiment and the slight effect of allowing for the reverse reaction. The results are as satisfactory as could be expected in view of the limits of error of the colorimetric method, the slight



changes in  $p_H$  accompanying the reaction and the simultaneous irreversible destruction of creatine and creatinine. In solutions alkaline to  $p_H$  3 the system is significantly reversible. The appropriate velocity equation may be put in the form

$$k_1 + k_2 = \frac{1}{t} \ln \frac{K a}{K a - (K + 1)x} \quad \dots(1),$$

where  $k_1$  is the monomolecular velocity constant for the hydration of creatinine,  $k_2$  is the monomolecular velocity constant for the dehydration of creatine,  $K = k_2/k_1$ , while  $a$  and  $x$  have their usual significance.

If  $K$  be known, this equation may be solved for  $k_1$  and  $k_2$ . Edgar and Shiver give the following relation for  $K$  (when the reactants are not measurably dissociated, i.e. in unbuffered solution)

$$\log K = -\frac{1084}{T} + 3.3652.$$

When this is solved for a temperature of  $30^\circ$  the value of  $K$  is given as 0.6125. According to the same authors  $K$  is related to  $[H^+]$  by an equation which (when  $k'$  values are substituted for  $k_2$  values) takes the form

$$K = 0.6125 \frac{k''[k' + [H^+]]}{k'[k'' + [H^+]]} \quad \dots(2),$$

where  $k'$  is the dissociation constant of creatinine and  $= 1.90 \times 10^{-5}$ , and  $k''$  is the dissociation constant of creatine  $= 2.40 \times 10^{-3}$ . From equation (2) we have calculated the values of  $K$  at the various  $p_H$  values of our reaction mixtures. When these are inserted in equation (1) together with the corresponding velocity data, the term  $k_1 + k_2$  is found to be reasonably constant within a single velocity experiment. One such result is summarised in the second part of Table IV, in which values of  $k_1 + k_2$  are contrasted with the values of  $k_2$  calculated as a monomolecular velocity constant. Finally,  $k_1$  and  $k_2$  have been calculated for each experimental  $p_H$  and the results are assembled in Table V. This Table is restricted to the same series of experiments as are shown in Fig. 1, while in Fig. 2 the values of  $k_1$  and  $k_2$  have been derived from two series of observations on the rate of dehydration of creatine and one series on the rate of hydration of creatinine. The curves have been further extended into the extremes of acidity and alkalinity by the rough calculation of the values of  $k_1$  and  $k_2$  from the observations of Edgar and Wakefield and of Hahn and Barkan respectively. These involve an uncertain temperature correction and can only be regarded as approximate.

Restricting further discussion to the range of  $p_H$  covered by our own observations the chief point of interest is that although equation (2) satisfies the equilibrium data it is not adequate to define the velocity relations. That is to say, the individual velocity constants display a relation to  $[H^+]$  which is not apparent in the equilibrium constant. The particular relation is the retardation of both velocities alkaline to  $p_H$  3. Since this is not reflected in a change in the equilibrium constants the factors responsible must have the same influence upon the two reactions. Indeed, it may be surmised that the same factor is responsible for the changes in both  $k_1$  and  $k_2$  within this range.

Table IV. *Rate of dehydration of creatine (0.0106 M) 30°.*

$p_H$ 2.00: $K = \frac{[\text{creatinine}]}{[\text{creatine}]} = 62.3.$					
$t$	$a$	$x$	$k_2 = \frac{1}{t} \ln \frac{a}{a-x}$	$k_1 + k_2 = \frac{1}{t} \ln \frac{Ka}{Ka - (K+1)x}$	
25	100	1.97	$80.0 \times 10^{-5}$	$80.0 \times 10^{-5}$	
75		5.58	$76.4 \times 10^{-5}$	$76.4 \times 10^{-5}$	
125		9.12	$76.4 \times 10^{-5}$	$76.4 \times 10^{-5}$	
170		12.25	$76.8 \times 10^{-5}$	$76.8 \times 10^{-5}$	
385		24.33	$72.5 \times 10^{-5}$	$73.1 \times 10^{-5}$	
865		46.18	$71.5 \times 10^{-5}$	$72.9 \times 10^{-5}$	
1346		59.25	$66.7 \times 10^{-5}$	$68.3 \times 10^{-5}$	
2017		76.92	$72.7 \times 10^{-5}$	$75.2 \times 10^{-5}$	
$p_H$ 3.77: $K = 5.673.$					
25	100	7.29	$304.0 \times 10^{-5}$	$350.0 \times 10^{-5}$	
50		15.39	$334.0 \times 10^{-5}$	$398.0 \times 10^{-5}$	
75		21.77	$327.0 \times 10^{-5}$	$393.0 \times 10^{-5}$	
125		31.93	$308.0 \times 10^{-5}$	$377.0 \times 10^{-5}$	
170		38.17	$283.0 \times 10^{-5}$	$350.0 \times 10^{-5}$	
385		64.62	$269.0 \times 10^{-5}$	$370.0 \times 10^{-5}$	
695		79.98	$232.0 \times 10^{-5}$	$405.0 \times 10^{-5}$	
1130		83.33	$159.0 \times 10^{-5}$	$347.0 \times 10^{-5}$	
1896		85.00	$99.0 \times 10^{-5}$	$370.0 \times 10^{-5}$	

Table V.

$p_H$	$K$ from equations of Edgar and Shriver	$k_1 + k_2 = \frac{1}{t} \ln \frac{Ka}{Ka - (K+1)x}$	$k_1$	$k_2$	$k_2$ calculated from equation (3)
1.37	73.1	$53.0 \times 10^{-5}$	$0.7 \times 10^{-5}$	$52.3 \times 10^{-5}$	$19.6 \times 10^{-5}$
2.00	62.3	$74.0 \times 10^{-5}$	$1.17 \times 10^{-5}$	$72.8 \times 10^{-5}$	$71.4 \times 10^{-5}$
2.83	29.8	$242.0 \times 10^{-5}$	$7.85 \times 10^{-5}$	$234.0 \times 10^{-5}$	$22.6 \times 10^{-5}$
3.77	5.673	$373.0 \times 10^{-5}$	$47.0 \times 10^{-5}$	$326.0 \times 10^{-5}$	$311.0 \times 10^{-5}$
4.64	1.335	$311.0 \times 10^{-5}$	$133.0 \times 10^{-5}$	$178.0 \times 10^{-5}$	$207.0 \times 10^{-5}$
5.63	0.086	$129.0 \times 10^{-5}$	$76.5 \times 10^{-5}$	$52.5 \times 10^{-5}$	$55.3 \times 10^{-5}$
6.86	0.614	$71.4 \times 10^{-5}$	$44.2 \times 10^{-5}$	$27.2 \times 10^{-5}$	$18.2 \times 10^{-5}$
7.62	0.613	$46.1 \times 10^{-5}$	$28.6 \times 10^{-5}$	$17.5 \times 10^{-5}$	$15.9 \times 10^{-5}$
8.49	0.613	$38.5 \times 10^{-5}$	$23.9 \times 10^{-5}$	$14.6 \times 10^{-5}$	$15.5 \times 10^{-5}$
9.54	0.613	$41.5 \times 10^{-5}$	$25.7 \times 10^{-5}$	$15.8 \times 10^{-5}$	$15.5 \times 10^{-5}$

It has been possible to evolve equations based on equation (2) but involving three empirical constants which define with a fair degree of accuracy the relations of  $k_1$  and  $k_2$  to  $p_H$  within the range 2 to 10. They do not cover the changes in  $k_1$  and  $k_2$  in strongly acid and alkaline solutions.

The equations are

$$k_1 = \frac{A'k'[C' + [H^+]]}{[k' + [H^+]][C' + [H^+]]}, \quad k_2 = \frac{A''k''[C' + [H^+]]}{[k'' + [H^+]][C' + [H^+]]} \quad \dots\dots(3),$$

where  $A' = 3.68 \times 10^{-3}$ ,  $C' = 0.8 \times 10^{-6}$  and  $C = 1.9 \times 10^{-5}$ ,  $A'' = 2.25 \times 10^{-3}$ .

It will be observed that, since  $K = k_2/k_1$ ,  $K$  becomes

$$0.6125 \frac{k''[k' + [H^+]]}{k'[k'' + [H^+]]}.$$

This is identical with equation (2).

Equations of the sort developed above have little merit other than the approximate summary of a mass of data. In particular, one must be very cautious in assigning any material significance to the various empirical constants. One point cannot, however, be overlooked. The value of  $C$  is identical

with the dissociation constant of creatinine. It is difficult to see in what way the dissociation of creatinine can affect the intrinsic velocity of dehydration of creatine. A possible explanation might follow the assumption that there was involved in the reactions a tautomer having a constant similar to creatinine.  $C'$  might then be regarded as a second constant of this structure or as indicating the participation of yet another intermediary.  $A'$  and  $A''$  are merely the values which  $k_1$  and  $k_2$  would have were they determined only by the concentration of undissociated molecules of creatine and of creatinine respectively.

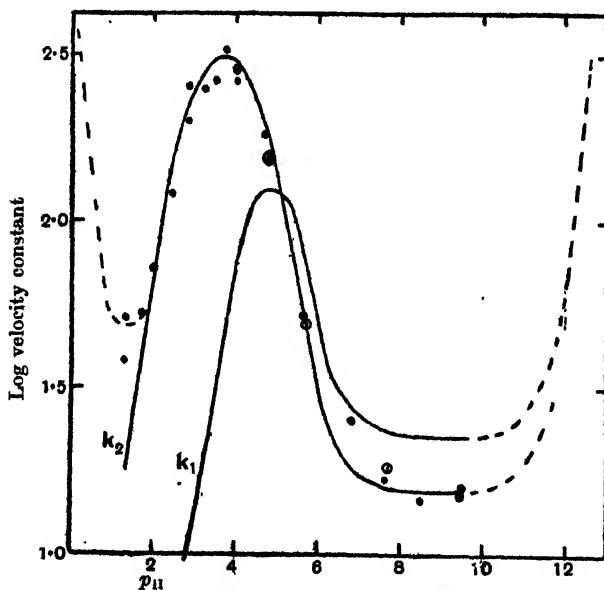


Fig. 2. Relation of velocity constants to  $p_H$ .

● Observed  $k_2$  from rate of dehydration of creatine.

○ Observed  $k_1$  from rate of hydration of creatinine.

Observed values of  $k_1$  are not inserted as they depart from their curve to the same extent as the corresponding values of  $k_2$ .

One final point of biological interest may be mentioned. Hahn and Meyer [1923] found that at  $38^\circ$  in a solution of 0.5 % creatine of  $p_H$  7.01 there appeared an amount of creatinine corresponding to 1.32 % of the total creatine in 24 hours. They calculate that the daily excretion of creatinine in the urine of an adult man corresponds to 1.33 % of the total creatine of his body. They suggest, therefore, that it is unnecessary to seek beyond the spontaneous dehydration of creatine for the origin of the creatinine of the urine. It follows from this view that the output of creatinine in the urine is governed only by the active mass of creatine in the muscles, by the temperature and by the  $p_H$  of the muscle. Hahn and his associates have made an attractive case for this simple hypothesis. The data of the present paper give a general confirmation of the above calculation. The velocity constant of the dehydration

of creatine at  $p_H$  7.2 and  $30^\circ$  is  $23 \times 10^{-5}$ . Applying the temperature correction of Edgar and Wakefield we arrive at a value of  $43 \times 10^{-5}$  at  $38^\circ$ . This corresponds to the dehydration of 1.03 % of the active mass of creatine in 24 hours. This figure—somewhat below that of Hahn and Meyer—is sufficient to account for the daily output of creatinine provided the active mass of creatine in living muscle is as great as 0.5 %. Evidence continues to accumulate, however, that this is an exaggerated value. It seems probable that only a small proportion of the total creatine which can be extracted from muscle by chemical means is in the free state in the living tissue. Unless the improbable assumption be made that combined creatine suffers dehydration as readily as when in the free state the argument of Hahn and Meyer cannot be sustained. It could then only be upheld were the demonstration made that the factors which have been shown to retard the velocity on the alkaline side of  $p_H$  3 were partially suppressed in living muscle. It would be necessary for the apparent constant  $C$  to be diminished or the constant  $C'$  to be increased to an extent corresponding to the ratio of free creatine to total creatine in muscle. Upon this possibility there is no evidence.

#### SUMMARY.

1. Determination has been made of the apparent dissociation constant (uncorrected for activity) of creatinine at  $15^\circ$ ,  $25^\circ$  and  $30^\circ$  and of the first dissociation constant (uncorrected) of creatine at  $17^\circ$ ,  $25^\circ$  and  $30^\circ$ .

2. The velocity constants of the reversible system creatine-creatinine have been determined at  $30^\circ$  over the  $p_H$  range 2 to 10, and have been related to  $[H^+]$ , the dissociation constants of the reactants and certain empirical constants.

#### REFERENCES.

- Bjerrum (1923). *Z. physikal. Chem.* **104**, 147.  
Bronsted (1923). *Rec. trav. chim.* **42**, 718.  
Cannan and Knight (1927). *Biochem. J.* **21**, 1384.  
Clark (1922). The determination of hydrogen ions (Baltimore).  
Eadie and Hunter (1926). *J. Biol. Chem.* **87**, 234.  
Edgar and Hinegardner (1923). *J. Biol. Chem.* **56**, 881.  
Edgar and Shiver (1925). *J. Amer. Chem. Soc.* **47**, 1179.  
Edgar and Wakefield (1923). *J. Amer. Chem. Soc.* **45**, 2242.  
Hahn and Barkan (1920). *Z. Biol.* **72**, 25, 305.  
Hahn and Fasold (1925). *Z. Biol.* **82**, 473.  
Hahn and Meyer (1923). *Z. Biol.* **78**, 91.  
Hunter (1928). Creatine and creatinine. (Monographs on biochemistry. London).  
McNally (1926). *J. Amer. Chem. Soc.* **48**, 1003.  
Simms (1926). *J. Amer. Chem. Soc.* **48**, 1239.  
Wood (1903). *J. Chem. Soc.* **83**, 568.

## CXVI. GUANIDINE STUDIES.

### II. DISTRIBUTION OF GUANIDINES IN ACUTE GUANIDINE AND PARATHYROPRIVIA TETANIES.

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*(Received June 6th, 1928.)*

#### INTRODUCTION.

ALTHOUGH guanidines can be recovered from the urine of animals after injections of guanidine salts, recoveries are not complete, especially with large doses [Burns, 1916]. This loss of guanidine could follow either the retention of guanidine as such or the chemical alteration of the guanidine by the body. The prolonged twitchings of voluntary muscle after the injection of even small quantities of guanidine salts, or after parathyroidectomy, suggest the retention of active guanidine, although they may equally well be the result of some unadjusted disturbance merely initiated by the guanidine in the case of the guanidine injections, or by guanidine or some other substance in the case of parathyroidectomy. Accordingly determinations of the guanidine of five different tissues from normal and injected rabbits have been made.

#### EXPERIMENTAL.

A modification of the extraction method previously described for invertebrate tissue [Ellis, 1928] was used for the separation of the guanidines which subsequently were reclaimed by adsorption with blood-charcoal in an alkaline medium, and determined colorimetrically by the methods of Marston [1924] and Weber [1927].

The tissues were taken from the animal as quickly as possible and weighed quantities placed directly in 5 to 10 volumes of alcohol, and immediately finely minced. After 18 hours the alcoholic mixture was extracted in a Soxhlet apparatus for several hours over a water-bath. To avoid adsorption no filter-paper thimbles or retainers were used. The alcoholic extract was evaporated to dryness under reduced pressure at about 90°, the residue taken up in a small quantity of warm water, diluted to 100 cc. with water after cooling, and protein was precipitated by adding 10 cc. of 2N/3 sulphuric acid and 7 cc. of 10 % sodium tungstate solution. After an hour the whole was filtered, approximately 1 g. of blood-charcoal (purified by acid) added to the filtrate, and the

mixture again filtered after about 5 minutes. A measured quantity (as large as possible) of this filtrate was made barely alkaline to litmus with 10 % sodium hydroxide and approximately 1 g. of blood-charcoal added for the adsorption of the guanidines. After 5 minutes the fluid was filtered off and discarded. The filter-paper and charcoal were drained quite free from fluid, and placed in the flask which had contained the alkaline mixture before it was filtered (to avoid loss of guanidines on the inside of the flask), together with 25 cc. of absolute alcohol. Sufficient hydrochloric acid (a few drops) was added to make the mixture distinctly acid to litmus, and the flask, securely stoppered, set aside for 18 to 24 hours. The charcoal was then filtered off, a measured quantity of the filtrate evaporated to dryness at 90°, and the residue taken up in water. Aliquot parts of this solution were used for the determination of guanidine [Marston, 1924; Weber, 1927], and of total and preformed creatinine [Folin, 1922]. By means of these two creatinine readings the proper corrections of the guanidine determinations were made [Weber, 1927; Major and Weber, 1927].

Total and preformed creatinine determinations were also made on a portion of the first acid filtrate after the removal of the proteins before charcoal was added, in order to obtain the creatine values for the entire sample. Blood-sugar values were secured from samples of fresh blood by the method of Hagedorn and Jensen [1923].

Young adult male rabbits were used in these tests, as the rabbit is readily poisoned by guanidine. Two sets of samples were taken; the first consisted of blood samples only, drawn from the ear vein of each rabbit after a 24-hour fast. From 5 to 7 cc. of blood were taken from each rabbit and the analyses of these samples are listed as "normals" in Table I. After taking the first blood samples the animals were fed for 4 days. No food was given on the 5th day, and the injections were made during the morning of the 6th day.

The blood samples after injection averaged about 18 cc. In addition to blood, the entire liver, without the gall-bladder, both kidneys, the entire brain, and a sample of voluntary muscle from the legs averaging 65 g. were preserved. Muscle was taken because of its obvious part in the tetany, liver and kidney because of their excretory functions, and the brain because it is an inactive tissue from a metabolic standpoint and because of its possible connection with the muscular tetany.

#### DISCUSSION.

The blood-guanidine values together with the readings for blood-sugar and blood-creatine have been assembled in Table I. The "normal" blood-guanidines of the rabbits varied from 0.086 mg. to 0.430 mg. per 100 cc. of blood, the average being 0.211 mg. The average of the rabbit guanidine values is slightly higher than those for human blood-guanidine [Major and Weber, 1927]. The blood-guanidines of the animals injected with guanidine or phenol lie within the limits of the normals, but their average falls below the general average, 0.176 mg. per 100 cc., of the normal and injected animals.

It is evident that high blood-guanidine at the time is not a requisite of acute tetany, for the guanidine-poisoned animals were in violent tetany when the blood samples were taken. There is however the possibility that the blood-guanidine may have risen in the injected rabbits at some time previous to the taking of the final blood sample, for Major and Weber [1927] report a rise in blood-guanidine from 0.108 to 0.530 mg. per 100 cc. in a dog during the first hour following the injection of 200 mg. of methylguanidine sulphate. The low blood-guanidine values in the acute stage of guanidine tetany suggest that the guanidine content of the blood may depend upon some part or parts of the body concerned with the elimination from the blood, or the destruction, of guanidine, rather than the actual amount present in the body or the stage of

Table I.

Rabbit no.	Treatment	Mg. per 100 cc. blood		Creatinine		
		Guanidine base	Sugar	total	preformed	Creatine
2	Normal	0.170	121	3.53	1.06	2.86
6	Normal	0.105	106	3.71	1.21	2.90
4	Normal	0.430	106	4.05	1.27	3.22
	5 cc. saline	0.200	126	3.86	1.01	3.51
5	Normal	0.086	87	3.94	1.43	2.90
	0.1 g. guan. hydrochlor.	0.087	31	4.73	1.69	3.52
3	Normal	0.380	84	3.84	2.01	2.12
	0.5 g. guan. hydrochlor.	0.097	39	2.61	1.30	1.51
1	Normal	0.108	103	4.02	1.52	4.00
	1 g. phenol	0.102	105	4.87	1.11	4.36
Average normal rabbits		0.211	104	3.85	1.33	3.07
Average guanidine rabbits		0.092	35	3.67	1.49	2.51
Phenol rabbit		0.102	105	4.87	1.11	4.36

*Rabbits 2 and 6, controls.* Blood samples taken from ears after 24-hour fast. No injections made.

*Rabbit 4, control.* Blood sample taken from ear after 24-hour fast; 4 days' full feeding; 5th day no food; 6th day, 10 a.m. injected 5 cc. of physiological saline, 2.30 p.m. animal killed by blow on head, samples taken immediately.

*Rabbit 5, guanidine.* Blood sample taken from ear after 24-hour fast; 4 days' full feeding; 5th day no food; 6th day, 10.10 a.m. injected 100 mg. guanidine hydrochloride (Merck) in 5 cc. physiological saline, little effect during 1st hour, tetany tremors began about 11.30 a.m. and increased steadily in violence and duration until 1 p.m. when respiration ceased, animal by this time in rigid tetany, decapitated immediately after cessation of respiration and a large sample of blood taken, heart still fibrillating when thoracic cavity was opened, muscles so rigid that they tore when attempt was made to bend leg, tissue samples taken at once.

*Rabbit 3, guanidine.* Blood sample from ear after 24-hour fast; 4 days' full feeding; 5th day no food; 6th day, 10.15 a.m. injected 500 mg. guanidine hydrochloride (Merck) in 5 cc. physiological saline, showed a little uneasiness after about 15 minutes, but became quiet again save for a slight twitching of ears, about 11 a.m. became restless again, suffering two short tetany spasms, at 11.30 a.m. gave one quick jerk, muscles became rigid, legs stiffened and were extended, and respiration ceased, decapitated immediately and a large blood sample collected, heart still fibrillating slightly when thoracic cavity was opened, muscles not so rigid as those in No. 5, tissue samples taken at once.

*Rabbit 1, phenol control.* Blood sample from the ear after 24-hour fast; 4 days' full feeding; 5th day no food; 6th day, 10.5 a.m. injected 100 mg. phenol in 2 cc. physiological saline, 11 a.m. animal showed slight tremor, 2 p.m. slight tremor, injected 400 mg. phenol in 8 cc. physiological saline, 2.30 p.m. tremor increased to tetany, injected 500 mg. phenol in 10 cc. physiological saline, 3.30 p.m. tetany spasms frequent and violent, 4.45 p.m. animal lying prostrate, respiration feeble, tetany violent, decapitated and a large blood sample collected, other tissue samples taken at once.

muscular tetany. This view is strengthened by the observations of Major and Weber [1927], who report that fluctuations in the blood-guanidine (from 0.140 to 0.350 mg. per 100 cc. and back again in the course of an hour) are not uncommon in dogs which have been injected with methylguanidine sulphate. If the actual value of the blood-guanidine be correlated with some factor other than that of muscular tetany alone, the lack of agreement in the guanidine values reported from parathyroidectomised animals by various writers can be readily understood.

The phenol rabbit, a tetany control against the guanidine rabbits, showed little change in blood-guanidine despite severe and prolonged tetany.

The guanidine rabbits developed marked guanidine hypoglycaemia, but neither the blood-creatine nor the blood-creatinine showed any definite correlation with guanidine poisoning.

The guanidine and creatine contents of the tissues are given in Tables II and III. The muscle-guanidines of the tetany animals were low, both actually and relatively, being but 12 % and 24 % of the muscle-guanidine of the normal rabbit. These muscle-guanidine values of the tetany rabbits were of the same magnitude as the blood-guanidine values, although actually the muscle-guanidine in each tetany animal was higher than the blood-guanidine taken at the same time, so that as far as equilibrium is concerned there could have been a movement of guanidine from the muscle tissue into the blood. This again suggests that the actual blood-guanidine value depends upon other factors than the guanidine content of the muscle alone. The presence of a large amount of guanidine in the muscles is evidently not essential to the maintenance of guanidine tetany.

Table II.

Guanidine and creatine: mg. per 100 g. tissue.

Rabbit no.	Treatment	Muscle.	Guanidine	Creatine
4	5 cc. saline		0.999	311.61
5	0.1 g. guan. hydrochlor.		0.124	202.10
3	0.5 g. guan. hydrochlor.		0.145	387.04
1	1 g. phenol		0.241	332.73
		Liver.		
4	5 cc. saline		0.625	16.50
5	0.1 g. guan. hydrochlor.		3.700	13.79
3	0.5 g. guan. hydrochlor.		6.340	12.44
1	1 g. phenol		0.161	12.40
		Kidney.		
4	5 cc. saline		Trace?	81.24
5	0.1 g. guan. hydrochlor.		0.852	102.10
3	0.5 g. guan. hydrochlor.		4.061	243.38
1	1 g. phenol		0.438	206.55
		Brain.		
4	5 cc. saline		Trace?	52.17
5	0.1 g. guan. hydrochlor.		0.600	11.28
3	0.5 g. guan. hydrochlor.		1.781	26.19
1	1 g. phenol		Trace?	39.31



The livers of the guanidine tetany rabbits were very rich in guanidine as compared with the livers from either the control or the "phenol control" rabbits, which suggests that the liver is concerned with the excretion or destruction of guanidines, or possibly both. The guanidine recoveries from the kidneys of the guanidine tetany rabbits were also high, both actually and relatively; this is not surprising however as guanidine is excreted as such in the urine.

The amounts of guanidine present in the brains of the normal and "phenol control" animals were too small to be determined, it being doubtful whether there was any guanidine in the brain tissue of these animals which could not be accounted for by the traces of blood which were unavoidably present, but the quantities of guanidine present in the brains of the guanidine tetany animals were large and, indeed, many times greater than either the muscle- or blood-guanidines of these same animals. The finding of guanidine in the brain is of interest in connection with the maintenance of the prolonged tetanies of guanidine poisoning, and points to an affinity of the brain tissue, and possibly of other nervous tissue, for guanidine. In view of the experiments of Fühner [1907], Meighan [1917], and Grant [1920], all of which show that guanidine acts on the nerve endings, the continued guanidine tetanies of muscles in which the actual guanidine content was low could be accounted for by the nerve endings taking up quantities of guanidine sufficiently large to produce marked activity in them, yet so small that as a part of the total guanidine of a large mass of muscle the guanidines contained in these nerve endings would make no appreciable difference in the determination of the muscle-guanidine.

Considering all the tetany animals together, both guanidine and phenol, there was a slight fall in liver-creatine, a more distinct fall in brain-creatine, and a very definite rise in kidney-creatine, associated with the tetany condition, whilst the muscle-creatine values varied about the normal. As the total creatine in the body is known to rise after some forms of muscular activity the high kidney creatine in these tetany animals may have been correlated with the severe tetanies which these animals experienced, although the high kidney creatine might also be interpreted as favouring the view advanced by Jaffé [1906] and Noël Paton [1925] that guanidine may be acetylated to form creatine. The urine was not analysed, so it is not known whether creatine, although found in considerable quantity in the kidneys, was actually being excreted in quantity by these guanidine-poisoned rabbits, and, as Jaffé [1906] did not find a rise in the creatine of the urine following the injection of methyl-guanidine, it is possible that the kidney-creatine found in these guanidine-tetany animals was not being-excreted.

From the guanidine values for the tissues it is possible to make a rather rough estimate of the disposal of the guanidine injected. Taking the average body weight of the guanidine tetany rabbits as 1 kg., rabbit No. 5 received 10 mg. guanidine hydrochloride (5.2 mg. guanidine base) per 100 g.

tissue, and rabbit No. 3, 26 mg. guanidine base per 100 g. tissue. If the injected guanidine were uniformly distributed throughout the body, and not destroyed or removed, approximately those values should have been recovered. It is not at all probable that such a uniform distribution of any soluble substance throughout the body would occur, but even considering these values to be the minimum required to account for the guanidine actually injected they greatly exceed any value found by analysis. The results of the present analyses, therefore, seem to substantiate the findings of Gergens and Baumann [1876] and others, that a considerable portion of the guanidine injected is changed in the body. It must be added however that the presence of considerable quantities of guanidine in the livers of the guanidine-tetany animals suggests the possibility that guanidine may be excreted through the bile (discussed in a following paper). Sharpe [1920] found guanidines in the faeces of children suffering from tetany, and if these guanidines were excreted they may have reached the intestine in the bile.

Table III. *Parathyroidectomised cats.*

Distribution of guanidine in the tissues: mg. per 100 g. (or cc.).

Cat	Treatment	Blood	Muscle	Liver	Kidney	Brain	Urine	Bile
A	None after operation	Trace	2.155	0.281	3.623	1.704	—	—
B	Dextrose and adrenaline	0.071	0.552	Trace	1.943	0.826	2.941	—
C	Insulin	0.110	1.193	0.012	1.198	0.433	—	None

For comparison with the tissues from the guanidine tetany animals analyses of tissues from three parathyroidectomised cats have been given in Table III. Cat A had no special treatment after the operation, and was killed on the 4th day after the operation when definite parathyroprivia tetany had developed. In cat B the tetany was controlled for 5 days (see last paper of this series) with injections of dextrose and adrenaline. The injections were then discontinued and the animal killed 24 hours later, *i.e.* 6 days after the operation, when tetany had returned. Cat C was given 20 units of insulin shortly after the first appearance of parathyroprivia tetany and was killed about 12 hours later just as it was about to die in acute tetany.

Perhaps the most striking feature of these analyses of tissues from the parathyroprivia tetany animals is the relatively high guanidine content of the brain, and if, as pointed out in the discussion of the guanidine-tetany animals, the guanidine content of the brain is in any way indicative of the guanidine content of the other nervous tissues, these analyses offer evidence in favour of the contention of Noël Paton *et al.* [1916] that the tremors and jerkings of the muscles are due to the action of guanidines on the central nervous system. The guanidine values from the tissues of cat A, the untreated parathyroidectomised animal, are in general similar to those of the guanidine tetany animals, except that the muscle guanidine in cat A is much higher. As the injections received by the other two cats had altered the sugar level in these animals the guanidine values from the tissues of cats B and C are not

strictly comparable with those of the guanidine tetany animals, but in connection with the relation of sugar to guanidine action (see last paper of this series) the low guanidine contents of the livers of cats *B* and *C* should be noted.

#### SUMMARY.

1. Analyses of blood, voluntary muscle, liver, kidney and brain of rabbits in acute guanidine tetany and acute phenol tetany were made and compared with normals for guanidine and creatine content.

2. The guanidine contents of the livers, kidneys and brains of animals in acute guanidine tetany were high.

3. The blood-guanidines and muscle-guanidines of animals at the point of death with acute guanidine tetany were lower than the blood- and muscle-guanidines of the control, showing that neither high blood-guanidine nor high muscle-guanidine is required to maintain guanidine tetany.

4. The presence of relatively large amounts of guanidine in the brains of the guanidine tetany animals offers a possible explanation of the maintenance of the prolonged tetanies regardless of the actual guanidine content of either the muscles or the blood during guanidine tetany.

5. No storage of guanidine exceeding or even equalling the average tissue guanidine, predicted on the basis of simple distribution of the guanidine injected, was observed.

6. The guanidine content of the brain of the untreated cat in acute parathyroprivia tetany was relatively high.

The writer is indebted to Professor D. Noël Paton and Professor E. P. Cathcart for their personal interest in this work and for the privileges of the Physiological Institute of the University of Glasgow; to Dr Alexander Watson who kindly made the injections; and to Mr A. Macfarlane for the blood-sugar determinations. The parathyroidectomies were performed by Professor D. Noël Paton.

#### REFERENCES.

- Burns (1916). *Biochem. J.* **10**, 262.
- Ellis (1928). *Biochem. J.* **22**, 353.
- Folin (1922). *Laboratory manual of biological chemistry* (New York).
- Fühner (1907). *Arch. exp. Path. Pharm.* **58**, 1.
- Gergens and Baumann (1876). *Arch. ges. Physiol.* **12**, 205.
- Grant (1920). *J. Physiol.* **54**, 79.
- Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.
- Jaffé (1906). *Z. physiol. Chem.* **48**, 430.
- Major and Weber (1927). *Arch. Int. Med.* **40**, 891.
- Marston (1924). *Austral. J. Exp. Biol. Med. Sci.* **1**, 99.
- Meighan (1917). *J. Physiol.* **51**, 51.
- Noël Paton (1925). *Glasgow Med. J.*, December 1.
- et al. (1918). *Quart. J. Exp. Physiol.* **10**, 315.
- Sharpe (1920). *Biochem. J.* **14**, 46.
- Weber (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 712.

## CXVII. GUANIDINE STUDIES.

### III. WATER CONTENT OF CERTAIN TISSUES DURING ACUTE GUANIDINE AND PARATHYROPRIVIA TETANIES.

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(Received June 6th, 1928.)

WHILE studying the distribution of injected guanidine in the tissues of guanidine tetany rabbits [Ellis, 1928], it was noted that the actual weights of the brains and kidneys from rabbits in acute guanidine tetany exceeded both the weights of these organs from control animals and the predicted weights for normal animals. A similar comparison of the weights of brains and kidneys from parathyroidectomised cats with normals showed the former to be greater than either the normal or predicted normal weights for cat brains and kidneys. In each group the control and experimental animals were quite comparable as regards body weight, and, as they were killed by drawing off the blood as completely as possible from the carotid artery (the organs being removed to

Table I. *Weights of organs (g.) from normal, injected and parathyroidectomised animals.*

Rabbits, average body-weight 1 kg.					
Animal no.	Experimental factor	Condition when killed	Brain	Kidney	Liver
4*	4 hours 30 minutes after injection 5 cc. physiol. saline	Normal	6.31	6.35	47.92
5*	3 hours after injection 0.1 g. guan. hydrochlor.	Acute tetany	12.34	15.00	42.46
3*	1 hour 15 minutes after injection 0.5 g. guan. hydrochlor.	Acute tetany	8.17	7.56	61.80
1*	6 hours 45 minutes after injection 1 g. phenol	Acute tetany	7.92	10.70	73.09
	Predicted weight for 1 kg. rabbit†		5.84	6.07	52.00
Cats, average body-weight, 2 kg.					
16	55 hours after parathyroidectomy	Tetany	29.30	15.20	65.25
17‡	127 hours after parathyroidectomy	Tetany	24.20	17.20	66.00
22‡	79 hours after parathyroidectomy	Tetany	23.95	18.50	50.40
	Predicted weight for 2 kg. cat†		20.80	7.40	59.20

\* Protocols given in preceding paper.

† Computed from tables of Skelton [1927].

‡ Tetany had been controlled previous to final tetany by injections, as compared with 16 which was uncontrolled.

sealed weighing vessels immediately after the blood was drained off), residual blood in the organs was not a disturbing factor. It was found by checking the gross weights, dry weights and ash, that the extra weight of the brains and kidneys from the tetany animals was due to the greater water content of these organs, and as may be seen in Table I the excess water amounted to 20 % or more.

Table II. *Water content of striated muscle from normal, injected and parathyroidectomised animals.*

Frogs, average body-weight 25 g.				Muscle		
Animal no.	Experimental factor	Condition when killed	Sample	Water %	Solid %	Ash %
7	Normal, 4 hours 15 minutes†	Normal	A	78.78	21.22	—
8	Normal, 6 hours 15 minutes†	Normal	A	79.35	20.65	—
2	3 hours 45 minutes after 30 mg. guan. carb.	Acute tetany	A	81.29	18.71	—
5	3 hours 45 minutes after 30 mg. guan. hydrochlor.	Acute tetany	A	81.51	18.49	—
4	5 hours after 15 mg. guan. carb.	Acute tetany	A	82.48	17.52	—
1	5 hours 30 minutes after 15 mg. guan. hydrochlor.	Acute tetany	A	80.98	19.02	—
6	5 hours 45 minutes after 0.2 g. guan. dextrose	Acute tetany	A	81.49	18.51	—
15	3 hours 15 minutes after 0.4 g. dextrose and 10 mg. guan. carb.	No tetany, sugar-poisoned	A	77.57	22.43	—
Rabbits, average body-weight 1 kg.						
4	4 hours 30 minutes after 5 cc. physiol. saline	Normal	B	78.81	21.19	5.43
5	3 hours after 0.1 g. guan. hydrochlor.	Acute tetany	B	86.11	13.89	5.47
2	1 hour 15 minutes after 0.5 g. guan. hydrochlor.	Acute tetany	B	80.49	19.51	5.96
1	6 hours 45 minutes after 1 g. phenol	Acute tetany	B	77.59	22.41	5.34
Rats, average body-weight 245 g.						
1	Normal; 5 hours 45 minutes after 2 cc. distilled water	Normal	A	73.09	26.91	—
			B	73.07	26.93	4.70
2	5 hours 45 minutes after 0.325 g. guan. hydrochlor.	Tetany	A	74.69	25.31	—
			B	75.45	24.55	4.58
5	1 hour 30 minutes after 0.275 g. guan. hydrochlor.	Tetany	A	76.05	24.95	—
			B	74.95	26.05	4.35
6	8 hours 15 minutes after 0.225 g. guan. hydrochlor.	Tetany	A	81.27	18.73	—
			B	75.55	24.45	—
Cats, average body-weight 2 kg.						
16	55 hours after parathyroidectomy	Tetany	B	78.62	21.38	—
17*	127 hours after parathyroidectomy	Tetany	B	77.84	22.16	—
22*	79 hours after parathyroidectomy	Tetany	B	78.51	21.49	—
25	Normal, control	Control	B	76.14	23.86	—

A Gastrocnemius muscle.

B Leg muscles collectively.

\* Tetany controlled by injections previous to final tetany.

† Uninjected animals kept for the time stated under the same conditions as the injected animals, as a control for the water loss by the animal as a whole.

To ascertain more exactly the extent of this change in water content the percentage values for water and solids, and in some cases the ratio of ash to solids, were determined for blood and striated muscles from normal, injected and parathyroidectomised animals.

Table II shows that the water content, compared with the normals, was higher and the total solids lower in the muscle from all tetany animals in which the tetany was induced by guanidine injections or parathyroidectomy. The ratio of ash to solids however was practically the same for both control and experimental animals, indicating that the change in the water-solids ratio in the tetany animals was due to an increase in the water content rather than to a loss of salts. The extra water taken up by the brain, kidneys and muscles during the guanidine tetany could have come from any of several sources including the blood, and the series of water-solids determinations, although small, for blood from three of the rats used (Table III), indicates that the blood is concerned, for the percentages of water were lower and of solids higher in the bloods of the tetany animals than in the normal.

The changes in the water-solids ratio in certain tissues during acute guanidine and parathyroprivia tetanies, particularly the rise in the water content of the muscles and the fall in the water content of the blood in guanidine tetany, suggest that calcium salts, which are known to reduce both guanidine and parathyroprivia tetanies [Fühner, 1906; György and Vollmer, 1922], may effect their action in part by withdrawing water from the muscles. Skelton [1927] found that the administration of calcium chloride to normal animals decreased the water content of the muscles and increased that of the blood. Such action by calcium salts would at least tend to restore the water balance in animals with guanidine and parathyroprivia tetanies and might afford opportunity for the movement of various substances along with the water withdrawn from the muscles.

Whether the increase in water content of these tissues during guanidine and parathyroprivia tetanies was one of the causes of the tetany or whether it was merely a corollary of the tetany was not determined. The muscles from frog No. 15 in which the guanidine tetany was intentionally prevented by an injection of dextrose, and those from the phenol-tetany rabbit did not show a rise in water content. There also seemed to be an indication that the increase in water content of the muscles during guanidine tetany was to some extent correlated with the severity and duration of the tetany. These observations collectively favour the view that the change in water content follows the tetany.

Table III. *Water content (%) of blood from rats listed in Table II.*

Rat no.	Sample 1	Sample 2	Average	Condition of animal when killed
1	81.20	81.80	81.50	Normal
2	75.97	77.80	76.88	Tetany
5	78.13	75.32	76.72	Tetany

In connection with the analytical procedure it should be noted that the changes in the water-solids ratio found in tissues from animals in guanidine and parathyroprivia tetanies have a bearing on the determination of blood- and tissue-guanidines; for the ratio of guanidine to tissue solids in those tissues which took up water, *i.e.* muscle, brain and kidney, would be higher than that derived from the values based on the weight of the normal tissues, while on the same basis the blood-guanidine in terms of normal blood would be too high. Corrections for the water content of the tissues however were not made in the preceding paper as the physiological significance of the excess water was not determined.

#### SUMMARY.

1. Brain, kidney and striated muscle from animals in acute guanidine and parathyroprivia tetanies were found to have higher water contents than the same tissues from normal control animals.
2. The ash-solids ratio was practically the same for muscle from the tetany animals and from the control normals, showing that the increase in weight of the tetany tissues was due to an actual increase in the water present rather than to a loss of salts.
3. Blood from rats in acute guanidine tetany was found to have a lower water content than normal rat blood.

#### REFERENCES.

- Ellis (1928). *Biochem. J.* **22**, 930.  
Fühner (1906). *Zentr. Physiol.* **20**, 838.  
György and Vollmer (1922). *Arch. exp. Path. Pharm.* **95**, 200.  
Skelton (1927). *Arch. Int. Med.* **40**, 140.

## CXVIII. GUANIDINE STUDIES.

### IV. CHANGES IN GUANIDINE ACTION AND IN PARATHYROPRIVIA TETANY PRODUCED BY DEXTROSE.

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*(Received June 6th, 1928.)*

GUANIDINE acting as a strong base is known to form compounds with several sugars including dextrose and galactose [Herzfeld, 1895; Morrell and Bellars, 1907], and this fact, together with the recovery of relatively large quantities of guanidine from the livers of animals in guanidine tetany [Ellis, 1928, 1], suggested the possibility that sugar may aid in the disposal of guanidines in the body. Some chemical and physiological tests of dextrose-guanidine have, therefore, been made and the effects of combined injections of guanidine carbonate and dextrose compared with those of guanidine carbonate alone.

The dextrose-guanidine was prepared by combining a saturated solution of dextrose in absolute alcohol with a strong solution of guanidine in absolute alcohol, the precipitated dextrose-guanidine being centrifuged down after the mixture had stood for 40 hours in an ice-box [Morrell and Bellars, 1907]. The guanidine solution was obtained by adding to an aqueous solution of guanidine carbonate slightly less than the amount of barium hydroxide required to precipitate completely the carbonate from the guanidine carbonate, after which the barium carbonate was filtered off and the filtrate evaporated to a very thick syrup (almost to dryness) under reduced pressure at 40°. This syrup was taken up in absolute alcohol and filtered to remove guanidine carbonate, the filtrate dropping directly into the saturated solution of dextrose in absolute alcohol. It is essential that absolute alcohol be used both to take up the guanidine and to dissolve the dextrose, and that all operations be performed in closed vessels so that moisture and carbon dioxide may be excluded. With these precautions the yield of dextrose-guanidine is very good. After the dextrose-guanidine had been centrifuged down it was washed with fresh absolute alcohol, recentrifuged, washed with dry ether, and then dried in a vacuum desiccator. The dextrose-guanidine was white in colour, very hygroscopic, and gave a clear solution in water with just a faint trace of yellowish-brown colour.



Morrell and Bellars [1907] assigned the formula  $3C_6H_{12}O_6 \cdot 2CH_5N_3$  to dextrose-guanidine and found it stable below  $50^\circ$ . They also noted that solutions of this substance are mutarotatory and that the rotation decreased during the first few days after the dextrose-guanidine was prepared, even if it were kept as dry powder, although it did not change in composition as determined by analysis.

A series of colorimetric determinations was made to ascertain the amounts of guanidine base in various quantities of dextrose-guanidine capable of giving the nitroprusside reaction. For these tests the various quantities of dry dextrose-guanidine were each taken up in 5 cc. of distilled water, the colour reaction obtained by means of the Marston [1924] reagent as modified by Weber [1927], and the readings made against known quantities of guanidine carbonate and guanidine hydrochloride. The results are summarised in Table I. The determinations show first of all that only a small portion of the guanidine base entering into the composition of dextrose-guanidine is capable of reacting with the nitroprusside reagent, which is very sensitive to minute quantities of guanidine salts and does not react with dextrose. The amount of dextrose-guanidine reacting as guanidine base is not proportional to the amount of dextrose-guanidine in solution, for the more dilute solutions gave relatively higher guanidine values. The age of the preparation was also a factor, for, after 24 hours in the desiccator as a dry powder, the portion of the dextrose-guanidine reacting with the nitroprusside reagent was still smaller. As controls against the guanidine determinations, samples containing 117 mg. of dextrose-guanidine per 100 cc. of distilled water were analysed for dextrose by the Hagedorn-Jensen method [1923] simultaneously with the first series of guanidine determinations; *i.e.* the sugar determinations are comparable with the first series of entries in Table I, and it was found that only about 57 % of the theoretical amount of dextrose in the dextrose-guanidine samples was determined by the Hagedorn-Jensen method. Dextrose-guanidine samples gave negative results when tested with the creatinine reagents of Folin [1922].

As the chemical tests indicated that guanidine did not react normally when combined with dextrose, a comparison of the toxicity of dextrose-guanidine, guanidine carbonate and guanidine hydrochloride was made. Six frogs of approximately equal weight were selected and placed in individual glass jars so that the factors of light, temperature and moisture were the same for all. After an hour for readjustment the animals were injected in the dorsal lymph sac, two with guanidine hydrochloride, two with guanidine carbonate and two with dextrose-guanidine, care being taken to disturb the animals as little as possible. They were then kept under continuous observation and the appearance of the several symptoms of guanidine poisoning recorded. Three points in the progress of the guanidine poisoning were noted in particular, the onset of continuous muscular tremor, the time at which the animal became prostrate and was unable to right itself after tetany spasms, and cessation of respiratory movements. The data from these animals (Table II) show that the dextrose-

guanidine used was much less toxic, weight for weight, than either guanidine carbonate or guanidine hydrochloride, and considerably less toxic than either of these two salts on the basis of the content of guanidine.

Table I. *Determinations of guanidine base in dextrose-guanidine samples.*

Immediately after drying				After 24 hours in desiccator as dry powder			
Dextrose-guanidine		Guanidine base		Dextrose-guanidine		Guanidine base	
Amount used (mg.)	Contained guanidine base* (mg.)	Amount found (mg.)	(%)	Amount used (mg.)	Contained guanidine base* (mg.)	Amount found (mg.)	(%)
1.17	0.210	0.079	37.8	1.18	0.211	0.046	21.8
2.34	0.420	0.144	34.3	2.35	0.421	0.062	14.8
3.51	0.630	0.195	31.0	4.70	0.842	0.046	5.5
				7.05	1.264	0.072	5.7
				11.75	2.106	0.084	4.0

\* Computed from the molecular formula for dextrose-guanidine as given by Morrell and Bellars [1907].

Table II. *Comparisons of toxicity of dextrose-guanidine with guanidine carbonate and guanidine hydrochloride.*

Frog no.	Body-weight (g.)	Substance injected	Guanidine base equivalent (mg.)	Time after injection		
				Onset of tremors hrs. mins.	Prostration hrs. mins.	Cessation of respiration hrs. mins.
1	24.5	15 mg. guan. hydrochlor.	7.8	1 26	3 06	5 31
4	31.5	15 mg. guan. carb.	9.84	0 55	1 00	4 30
5	26.5	30 mg. guan. hydrochlor.	15.6	0 25	3 05	4 35
3	26.5	100 mg. dextrose-guanidine	17.94	4 00	6 15	Recovered
2	22.0	30 mg. guan. carb.	19.67	0 20	0 46	3 40
6	26.5	200 mg. dextrose-guanidine	35.88	1 06	3 10	5 45

All injections were 1 cc. in volume. The dextrose-guanidine used had been kept as a dry powder for 48 hours in a desiccator.

Since no method was available by means of which the presence of dextrose-guanidine as such could be demonstrated in living animals, and as the real value to an animal of such a sugar-guanidine combination with reduced toxicity would lie in the formation of this combination within the body from more active guanidine compounds, combined injections of dextrose and guanidine carbonate were compared with injections of guanidine carbonate alone. In Table III the condensed data from a series of ten frogs are given. To allow for differences in the rates of absorption of the dextrose and the guanidine carbonate, the dextrose was injected 1 hour in advance of the guanidine in two frogs,  $\frac{1}{2}$  hour in advance in two, and simultaneously with the guanidine in two others. Large doses of dextrose were used to provide a liberal excess of dextrose above that required on a chemical basis to combine with the guanidine, if this combination were to take place within the body. As frogs will excrete dextrose very rapidly if kept in water (in another series of experiments the writer has found that 25 to 30 g. frogs will survive the injection of 1 to 1.5 g. of dextrose if placed at once in a large volume of fresh water), the frogs in this

guanidine and dextrose series were kept in individual glass jars containing only single moist filter-papers for the first 6 hours after the injections, to insure the presence in the body of a considerable portion of the injected dextrose during the period in which the guanidine poisoning symptoms should appear. The prompt appearance of the guanidine symptoms in the frogs which were given guanidine alone is in sharp contrast with the absence of guanidine symptoms during the first 5 hours in all frogs receiving guanidine and dextrose. With the exceptions of frog no. 18 which received its guanidine and dextrose simultaneously, and frog no. 15 which died 3 hours after the injection of guanidine, the other frogs receiving both dextrose and guanidine did not develop guanidine symptoms until they had remained in water for 12 hours or

Table III. *Comparison of dextrose and guanidine injections with injections of guanidine alone.*

Frog no.	Body-weight (g.)	Dextrose*		Guanidine-carbo-nate† (mg.)	Time after injection			Final condition
		Amount (mg.)	Time		Onset of tremors (hrs. mins.)	Prostration (hrs. mins.)		
13	29.0	400	1 hour before guanidine	10	26 30	None		Recovery at 72 hours
14	25.5	200	1 hour before guanidine	10	48 00	None		Recovery at 72 hours
15	30.2	400	$\frac{1}{2}$ hour before guanidine	10	None	None		Dead, 3 hours 15 minutes
16	24.0	200	$\frac{1}{2}$ hour before guanidine	10	48 00	None		Recovery at 72 hours
17	31.7	400	With guanidine	10	48 00	None		Recovery at 72 hours
18	28.5	200	With guanidine	10	5 00	None		Dead, 48 hours
19	32.0	None	—	10†	0 23	0 24		Recovery at 72 hours
20	36.7	None	—	10†	0 22	1 38		Dead, 18 hours
21	23.5	200†	—	None	None	None		Recovery at 72 hours
22	28.7	400†	—	None	None	24 00		Dead, 27 hours

\* Injected in 1 cc. of distilled water.

† Injected in 0.5 cc. of distilled water.

‡ Injected in 1.5 cc. of distilled water so that the total volume of fluid received by these animals would be the same as that received by the animals given two injections.

more, *i.e.* for 18 hours or more after the injection of the guanidine. Even frog no. 15 which presumably died from an overdose of dextrose (judging from the edematous condition of the body similar to that of other frogs killed with dextrose alone, *e.g.* frog no. 22) gave no evidence externally at least of guanidine poisoning. When placed in water the frogs which had received both guanidine and dextrose did develop guanidine tremors after 24 hours or more (see Table III) but without prostration, the tremors being much less severe than those in the frogs receiving only guanidine. In view of other experiments with dextrose alone it seems that the frogs which received both guanidine and dextrose lost dextrose rapidly, even after 6 hours in moist air, on being returned to water, and that the elimination of dextrose proceeded more rapidly than the elimination or destruction of the guanidine compounds. The protective value of the sugar against guanidine before the frogs were returned to water was evident however, and this was the point in question.

The mechanism of this relation between guanidine and dextrose in the body was not worked out in these preliminary experiments, but the relatively low toxicity of dextrose-guanidine offers an explanation if such sugar-guanidine compounds be formed in the body. It is also possible in the case of the frogs that changes in water balance may have been effected by the dextrose which offset those induced by guanidine poisoning and that the guanidine effects were counteracted in this way [see previous paper, Ellis, 1928, 2], for the muscles of frog no. 15 did not show an increase in the water content following the injection of dextrose and guanidine as did the muscles of frogs receiving guanidine alone. The work of Henderson [1910] must also be considered in this connection as presenting still another possibility. He found that the contractions and twitchings of isolated frog muscles caused by guanidine salts and by sodium oxalate were inhibited or prevented by immersing these muscles in solutions of glucose, mannitol and cane sugar, and suggested that the movement of the guanidine and the sodium oxalate into the muscle may be influenced by these sugar solutions.

As Paton and Findlay [1916] have favoured the view that parathyroprivia tetany results from an increase in the tissue-guanidines, the action of dextrose injections on parathyroidectomised cats was tried. Four parathyroidectomised cats were used, and each animal was kept until definite parathyroprivia tetany developed (usually on the third day after operation) before being injected. When the tetany became acute the first animal was given 20 units of insulin to lower the available sugar. This animal died in severe convulsions during the next 12 hours. The second and third parathyroidectomised cats were each given 10 cc. of sterile dextrose solution (20 %) intraperitoneally when the tetany became acute, and in each case the tetany disappeared and the animal showed marked improvement in condition during the following 6 to 8 hours. By repeating these injections once or twice a day these cats were kept free from tetany and in fair condition for 5 days, when the injections were terminated and the animals killed for analysis after being allowed to develop acute tetany again. The fourth cat was given an injection of adrenaline (1 cc. of 1 : 10,000) when the acute tetany stage was reached, and after an hour of marked depression the tetany disappeared and the condition of the animal was very good for the following 10 hours. The symptoms of tetany then gradually reappeared and the animal became prostrate again. A double injection of dextrose (20 cc.) was given and the animal recovered a second time. By alternating the adrenaline and dextrose injections this animal was kept free from tetany and in fair condition for 5 days, when it was allowed to develop acute tetany and was killed for analysis.

These tests on parathyroidectomised animals collectively favour the view that dextrose and guanidine are antagonistic, if guanidine be considered the causative agent for parathyroprivia tetany. If not, the correlation between the condition of tetany and the sugar level still remains, for the tetany became more acute when the sugar level was lowered by insulin, while the tetany was

in a large measure controlled by injections of dextrose or adrenaline, which raised the sugar level and maintained the animals for 3 or 4 days beyond the time at which uncontrolled parathyroidectomised animals usually die. It is interesting to add that Dr A. Watson, who has made certain histological studies of tissues from parathyroidectomised animals, informs me that the livers from animals in uncontrolled parathyroprivia tetany contained much glycogen.

#### SUMMARY.

1. Dextrose-guanidine was found to be less active both chemically and biologically than its equivalent of guanidine base.
2. Combined injections of dextrose and guanidine carbonate were less toxic than injections of guanidine carbonate alone.
3. By means of dextrose and adrenaline injections parathyroprivia tetany in cats was relieved, and these animals were maintained for 3 or 4 days beyond the time at which uncontrolled parathyroidectomised cats usually died.

#### REFERENCES.

- Ellis (1928, 1). *Biochem. J.* **22**, 930.  
— (1928, 2). *Biochem. J.* **22**, 937.  
Folin (1922). *Laboratory manual of biological chemistry* (New York).  
Hagedorn and Jensen (1923). *Biochem. Z.* **135**, 46.  
Henderson (1910). *Zentr. Physiol.* **24**, 519.  
Herzfeld (1895). *Z. ver. deutsch. Zuckerind.* **45**, 853.  
Marston (1924). *Austral. J. Exp. Biol. Med. Sci.* **1**, 99.  
Morrell and Bellars (1907). *J. Chem. Soc.* **91**, 1010.  
Paton and Findlay (1916). *Quart. J. Exp. Physiol.* **10**, 315.  
Weber (1927). *Proc. Soc. Exp. Biol. Med.* **24**, 712.

# CXIX. A METHOD FOR THE ESTIMATION OF THE SALT CONTENT FROM THE $p_H$ VALUE OF APPLE JUICE, AND SOME COMPARATIVE ANALYSES OF THE MINERAL CONTENT OF THE JUICE AND WHOLE APPLE.

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*(Received June 7th, 1928.)*

MEASUREMENTS of the hydrogen ion content of the juice of apples were undertaken by one of the authors (D. H.) some years ago in order to ascertain whether any sudden change in the value of this property marked the beginning of breakdown. The experiments were carried out over two seasons and no sudden change of  $p_H$  occurred, although alterations in the relation of this property to the titratable acidity were observed. These results could not be completely interpreted without the aid of mineral analysis, but now that the other author (J. W. B.) has elaborated methods for a complete mineral analysis of the apple, a comparison between salt content and  $p_H$  has been made possible, and may be usefully discussed.

It is shown below that if the  $p_H$  and titratable acidity of the juice of acid apples is measured, it is possible to obtain a fairly reliable idea of its salt content by comparison with the acidity and  $p_H$  of mixtures of malic acid and potassium malate of known concentration. Since these measurements can be carried out rapidly, while mineral analyses are long and laborious, this method of obtaining an approximate estimate of salt content is likely to prove to be of value, if the soluble salt contained in the expressed juice is a measure of that contained in the whole apple. Previous examinations of successive fractions of expressed juice have been shown to give little or no evidence of any retention of sugars or acids in the press residue, for the value of  $p_H$  in these fractions has been found to correspond with the titration, which indicates that the concentration of salts does not vary sufficiently to affect the hydrogen ion concentration appreciably [Haynes and Judd, 1919; Haynes, 1921]. More delicate methods of mineral analysis have, however, brought to light small differences in the juice expressed from the apple under different conditions, which suggest that the acid sap dissolves certain constituents of the cell wall. These observa-

tions, and the relation of  $p_H$  to mineral content, are discussed below under the following headings.

(1) The hydrogen ion concentration ( $p_H$ ) of mixtures of malic acid and potassium malate.

(2) Comparisons of the "equivalent potash" calculated from measurements of  $p_H$  and titratable acid in certain juices with the potash equivalent of the potash, magnesia and lime found by analysis in the same juices. The term "equivalent potash" is introduced to express the effect upon the  $p_H$  of all the cations present. It represents the amount of potash, combined as malate, which would be required to raise the  $p_H$  of a solution of malic acid, of concentration equal to that of the titratable acid present, to the observed value. In apple juice the "equivalent potash" is only slightly greater than the actual quantity of potash present, since the percentage of potash in the ash is very large compared with the percentages of lime and magnesia, the chief other cations present. The further assumption involved, that all the acid is present as malic acid, is discussed below.

(3) Titrations and  $p_H$  values of the juice of various apples at intervals during storage and their "equivalent content" of  $K_2O$ .

(4) A comparison between the mineral analysis of apples with that of their expressed juice, and the changes in the composition of the juice during a period of storage.

The methods used both for  $p_H$  determinations and mineral analyses have been already described [Haynes, 1925; Brown, 1926], but one point of difference may be noted. Measurements of  $p_H$  were made as before in a water-bath kept at a temperature of  $25^\circ$ ; as the whole apparatus could not be submerged a small difference of temperature usually existed between the parts in air and water; in the previous measurements<sup>1</sup> this was compensated by an alteration in the value of  $E$  in the factor  $\frac{E-\beta}{f(t)}$ ; in the present instance the apparatus was so arranged that for small differences of inside and outside temperature the correction became negligible.

(1) *The hydrogen ion concentration ( $p_H$ ) of mixtures of malic acid and potassium malate.*

In order to investigate the relation between the  $p_H$  and the salt content of the juice, a number of measurements of  $p_H$  were carried out in mixtures of malic acid and potassium malate of known composition. All the measurements were made in duplicate on solutions prepared from good commercial specimens of malic acid; the measurements were, however, confirmed by further observations made with solutions of recrystallised malic acid. The more concentrated acid solutions were made up and standardised by titration with phenolphthalein as indicator, the weaker solutions being obtained from these by dilution. Potassium malate solutions of concentration 0.2N, the strongest

<sup>1</sup> This difference was formerly erroneously attributed to incomplete saturation.

solutions used, were prepared by mixing equal volumes of carefully standardised solutions of malic acid and potassium hydroxide of double this strength; other solutions were obtained from these by dilution. The  $p_H$  values corresponding with the final concentrations of these various mixtures are shown in Fig. 1.

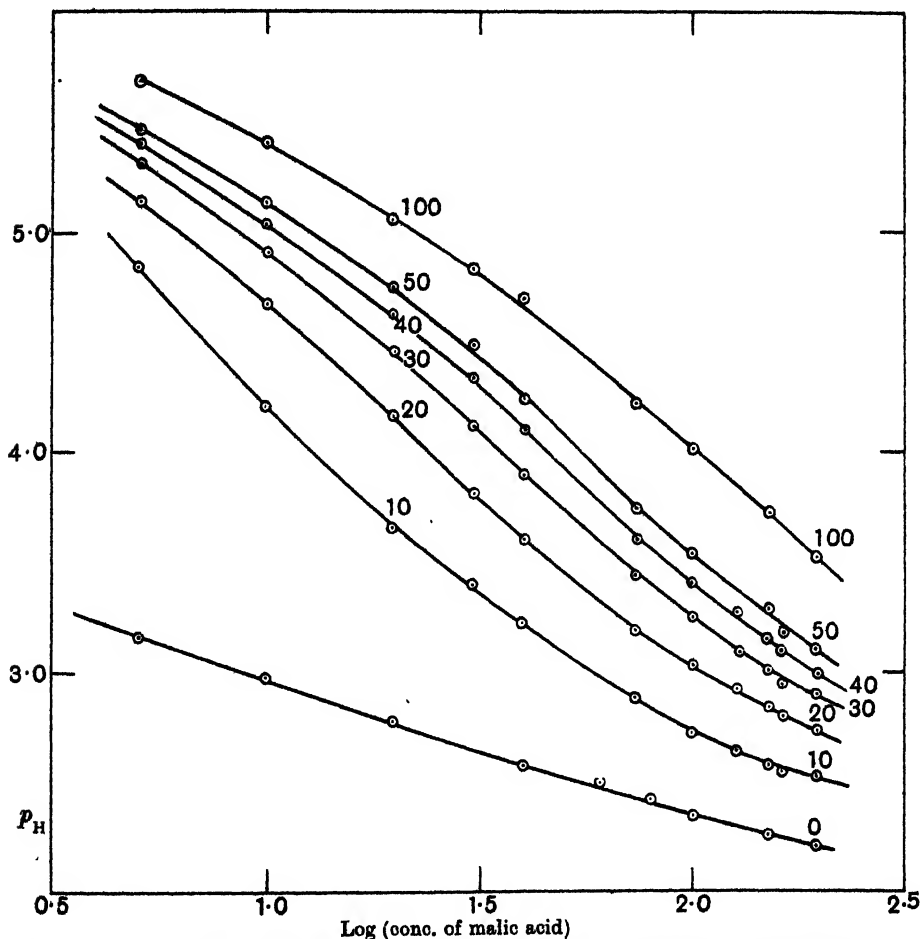


Fig. 1.  $p_H$  of mixtures of malic acid and potassium malate. The figures on the curves denote concentrations of K malate. Concentrations in mg. equivalents per litre.

The range of  $p_H$  covered is from 2.2 to 5.7, i.e. of hydrogen ion concentration from  $63 \times 10^{-4}$  to  $0.02 \times 10^{-4}$ , which is a considerably larger range than that shown by the juice. In Fig. 1  $p_H$  is plotted against the logarithm of the titratable acidity in order to make it evident that  $p_H$  approximates to a linear function of the acid concentration. The curves of Fig. 1 show very plainly the effect of the presence of salts upon the hydrogen ion concentration; they represent the results of the interaction of three constituents, malic acid, potassium malate and potassium hydrogen malate. In the first part of every curve for the solutions containing salt, the only acid present is potassium



hydrogen malate; the second part of the curve shows the buffer effect of this latter compound upon malic acid. It is evident that the buffer effect increases considerably as the proportion of salt increases. This is probably of some physiological importance, for there is a considerable difference between the salt content of different varieties of apples, and in the same variety grown on different soils or grafted on different stocks.

Before proceeding to a direct comparison between the salt content as estimated from measurements of  $p_H$  and titratable acidity, and that found by analysis, some further discussion of the fundamental assumptions involved is required. The presence of cations other than potassium has already been mentioned, and it has been pointed out that the joint effect of all cations may be estimated as "equivalent potash"; for in such a dilute solution as apple juice the salts of organic acids present are completely dissociated. This assumption is supported by the results of Table I, which show that where the amount of malic acid is very small, calcium malate produces very nearly the same effect as an equivalent quantity of potassium malate. This will also be true of the corresponding magnesium and other similar salts; the buffer effect of other cations can therefore be calculated with sufficient accuracy in terms of potassium.

Table I.  $p_H$  of mixtures of malic acid and calcium malate.

Concentration of calcium malate 10 mg. equivalents per litre.		
Malic acid mg. equivalents per litre	$p_H$	$p_H$ of similar solution containing potassium salt
10	3.97	4.21
50	3.00	3.12
100	2.72	2.74
150	2.55	2.57
200	2.45	2.48

The presence of acids other than malic acid has still to be considered and this is highly important, for the possibility that apple juice may contain some considerable quantity of these other acids constitutes the only serious difficulty in the use of the indirect method of estimating the salt content as described above, since the effect of the sugar and other non-electrolytes which the juice contains is so small that it may safely be disregarded. Although unfortunately the difficulty of estimating mixtures of organic acids has prevented a direct analysis of the quantities of these other acids present in the juices used, some information is available upon the general question. Citric acid appears to be the only organic acid other than malic acid that is present in any considerable quantity in apples. Franzen and Helwert [1923] found about 20% in the sample of apples which they examined; whether these apples were of any definite variety is unstated. More recently Nelson [1927] has found Winesap apples to contain only a trace of citric acid, and in York Imperial apples none could be detected. Fig. 2 shows the  $p_H$  of solutions of citric acid and potassium citrate, the corresponding values of a similar mixture of malates being given for

comparison. It will be observed that while solutions of citric acid are slightly more acid than malic acid solutions of equivalent concentration, mixtures of citric acid with its salts are slightly less acid. The effect of replacing malic by citric acid

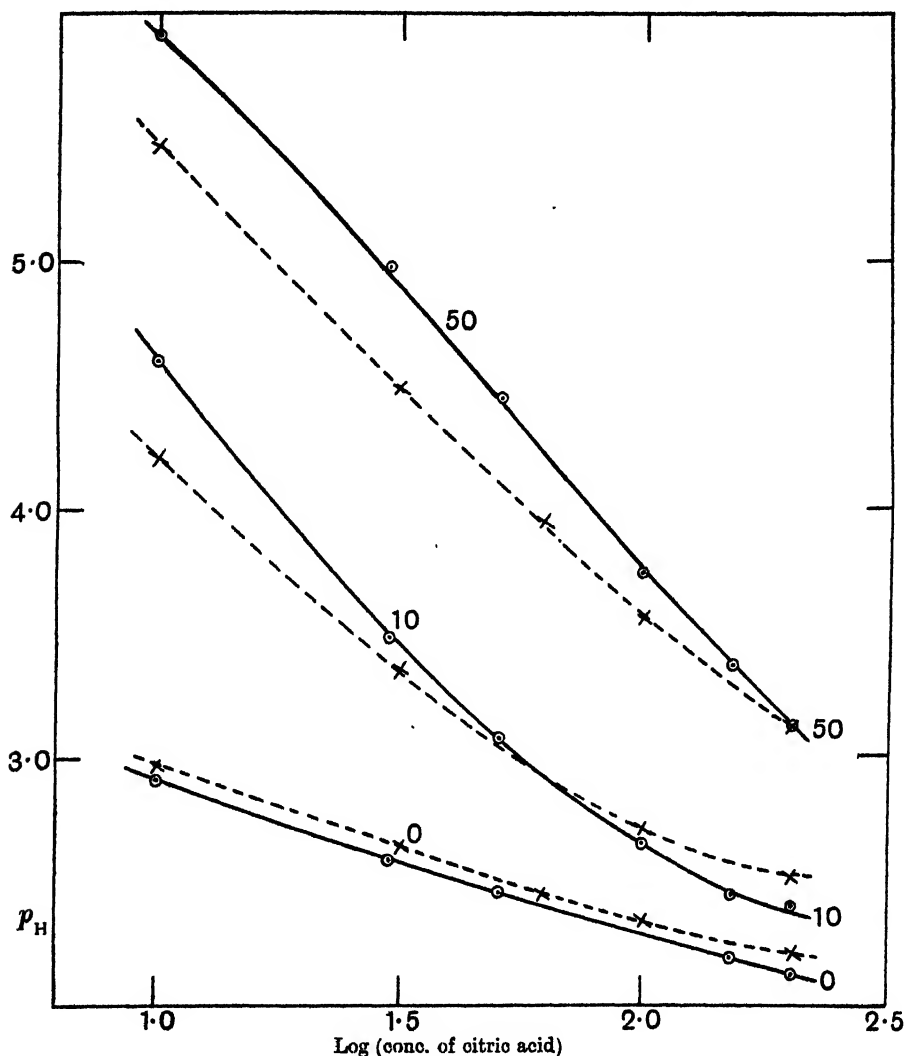


Fig. 2.  $p_H$  of mixtures of citric acid and potassium citrate. The figures on the curves denote concentrations of K citrate. Concentrations in mg. equivalents per litre. The dotted curves show the corresponding values for malate.

in apple juice must therefore be very small, especially as to mix acids is necessarily to reduce buffering and thus to increase acidity. The  $p_H$  of the mixtures of citric and malic acids and their salts must therefore approximate very closely to that of a simple mixture of malic acid and its potassium salt in equivalent concentrations.

The presence of strong mineral acids might be disregarded but for the general use of sulphate manures, as a result of which the juice may contain quantities of sulphuric acid which are not entirely negligible. Although it is obvious that the salts of mineral acids can be only very slightly affected by admixture with malic acid, a few experiments have been made which establish that within the range of concentrations dealt with in Fig. 1 the addition of potassium chloride to solutions of malic acid tends to increase acid reaction to a very small extent, while the addition of potassium sulphate tends to decrease it, though again very slightly; in the presence of potassium malate both effects tend to disappear. Hence, since the potassium or other cation combined with mineral acid is without buffer effect, it will not be estimated by the indirect method, and therefore the presence of sulphate will tend to produce low results. This is probably a principal cause of discrepancy between the "equivalent potash" as estimated by analysis and as calculated by the indirect method.

Some experiments carried out on the effect of phosphates on the  $p_H$  of mixtures of malic acid and potassium malate within the range of concentration usually found in apple juice (0.04*N* — 0.2*N* acid, and 3.0–3.75 mg. equivalents of potash per litre) showed that for the higher concentrations (0.05*N* acid and more) the effect of replacing potassium malate by an equivalent quantity of potash as phosphate (either the dihydrogen or monohydrogen phosphate) was to raise the  $p_H$  value; thus the calculated value of potash equivalent in the presence of phosphate is higher than the true potash content. The error is of the order of 5 %. In concentrations of acid of 0.05*N* and less, the phosphate has the opposite effect, and the calculated value of potash equivalent is therefore lower than the true value. When citric acid was substituted for malic acid, for the concentrations of acids used (0.08*N*, 0.1*N*, and 0.13*N*) the  $p_H$  was lowered by the presence of phosphate, rendering the calculated value of the potash equivalent lower than the true value. Thus when the titratable acid is of the order of 0.1*N* the effect of phosphate on the  $p_H$  of mixtures of citrates and malates and their acids will tend to be neutralised, and this will probably occur in acid apples, but in pears and sweet apples, which contain only a small percentage of total acid, the calculation of salt content from the  $p_H$  value may be seriously affected, and further investigation is necessary before the indirect method can be used in these slightly acid juices.

Another condition tending to an undervaluation of the soluble salt content is the production of soluble pectin. This when separated shows a very marked capacity for absorbing salts and may well exert a similar action in the natural medium, but the quantity of soluble pectin present is small and any removal of salts from the sphere of action brought about by its agency must be relatively unimportant.

The result of the foregoing discussion is therefore to justify the approximate estimation of equivalent potash present in apple juices from their titration and  $p_H$  values, where the titration is not too low. It must be remembered

that in this method all the cations are estimated together as "equivalent potash" so that no knowledge of the relative percentages of the different constituents is obtained, and that the result is only intended to be approximate, for it is affected by the different sources of error discussed in detail above. It is perhaps worth while to suggest that this method of analysis might be extended to non-acid biological material, by adding malic acid of known concentration to the solution of the ash, and measuring the  $p_H$ , but the method will probably be found to be most valuable if used for the preliminary examination of material upon which it is intended to make a detailed mineral analysis.

Table II. *Comparison between (a) mineral content of the whole apple and juice, (b) direct and indirect estimation of the mineral content of juice, using Lane's Prince Albert apples (East Malling Stocks), 1924.*

The figures represent percentages referred to fresh weight.												
Whole apple					Juice							
Estimation by analysis					Estimation by analysis			Indirect estimation from $p_H$ value and titration				
Stock	$K_2O$	$CaO$	$MgO$	Total "equiv. $K_2O$ "	$CaO$	$MgO$	Total (a) "equiv. $K_2O$ "*	mg.equiv. per litre	$p_H$	Difference		
										(alc. (b) "equiv. $K_2O$ ")	between (a) and (b) in g.	as % of (a)
type I	0.0983	0.0036	0.0068	0.1108	0.0050	0.0083	0.1262	249	2.72	0.116	0.010	7.9
type II	0.0104	0.0031	0.0071	0.1323	0.0040	0.0069	0.1333	257	2.78	0.141	0.008	6.0
type IX	0.0811	0.0032	0.0061	0.1008	0.0051	0.0087	0.1099	196	2.83	0.122	0.012	11.0

\* Using the  $K_2O$  figures for the analysis of the whole apple, since as is shown below the potash content of the juice and the whole apple is the same within the limits of error.

(2) *Comparisons of the "equivalent potash" calculated from measurements of  $p_H$  and titratable acid in certain juices with the potash equivalent of the potash, magnesia and lime found by analysis in the same juices.*

These comparisons are shown in Tables II and III and in one of the sets of analyses in Table VII. The calculated values were obtained as explained above by finding that point in Fig. 1 whose co-ordinates are the logarithm of the titration, and the  $p_H$  of the juice under examination. The concentration of potash in the corresponding malate mixture gives the value of the "equivalent potash" concentration of the juice. In any given value of  $p_H$  and acidity plotted on the graph of Fig. 1, the value of the "equivalent potash" is found from the ratio of the vertical distances of the point from the curves of concentration of "equivalent potash" between which the point lies. It has been found convenient in practice to make from Fig. 1 a table showing  $p_H$  and potash values for an increase of 0.05 in the logarithm of the titrations. The table gives differences of  $p_H$  corresponding to a rise of 10 mg. equivalents of potash, and average differences of  $p_H$  corresponding to increases of 0.01 of the logarithm of titration. With such a table values of "equivalent potash" can be easily and rapidly read.

Table II shows an analysis of Lane's Prince Albert apples grown upon various stocks. The direct and indirect analyses of "equivalent potash" agree fairly satisfactorily for the apples grown on stocks I and II. The analyses of

the juice of the apples grown on stock IX show a greater difference, which may possibly be significant, since type IX is a dwarfing stock of unusual character. The observation, however, needs confirmation before any deductions can be drawn from it, but if confirmed it would suggest a possible method by which some of the physiological differences of this nature might be investigated.

Table III. *Comparison between potash content found by analysis of the whole apple and by calculation from  $p_H$  and titration values in 30 individual Bramley's Seedling apples.*

K <sub>2</sub> O figures are given as percentages referred to fresh weight of apple.					
Log. titration mg. equiv. per litre	$p_H$	"Equiv. K <sub>2</sub> O" calc. from $p_H$ and titration	K <sub>2</sub> O found by analysis	Difference	
2.299	2.95	0.160	0.1175	0.042	
2.286	2.99	0.168	0.1170	0.051	
2.358	2.87	0.152	0.1167	0.035	
2.318	2.88	0.140	0.1094	0.031	
2.272	2.89	0.126	0.0969	0.029	
2.230	2.89	0.117	0.0965	0.020	
2.230	2.95	0.135	0.1120	0.023	
2.274	2.95	0.148	0.1060	0.042	
2.274	2.90	0.131	0.1074	0.024	
2.236	2.95	0.135	0.1303	0.005	
2.281	3.05	0.188	0.1148	0.073	
2.290	2.98	0.163	0.1150	0.048	
2.267	3.09	0.192	0.1216	0.070	
2.161	2.92	0.105	0.1280	0.021	
2.240	3.06	0.177	0.1690	0.008	
2.297	2.93	0.152	0.1357	0.016	
2.215	3.05	0.161	0.1215	0.039	
2.223	3.01	0.151	0.1000	0.051	
2.258	3.02	0.170	0.1455	0.024	
2.137	3.07	0.137	0.1371	—	
2.220	3.02	0.151	0.1600	0.009	
2.292	2.96	0.160	0.1381	0.022	
—	2.89	—	0.1155	—	
2.334	3.02	0.189	0.1782	0.013	
2.233	3.05	0.180	0.1353	0.045	
2.267	3.02	0.174	0.1319	0.042	
2.182	3.02	0.137	0.1128	0.024	
2.185	3.01	0.135	0.1500	0.015	
2.238	3.06	0.177	0.1629	0.014	
2.182	3.13	0.174	0.1211	0.053	

*Mean values.*

"Equivalent K <sub>2</sub> O" calculated from $p_H$ and titration	...	0.155
K <sub>2</sub> O found by analysis	...	0.127
Difference*	...	0.028
CaO and MgO as equivalent K <sub>2</sub> O†	...	0.0188
Total "equivalent K <sub>2</sub> O" by analysis	...	0.146
Difference from calculated value	...	0.009 or 6.2 %‡

\* Chiefly due to the inclusion of CaO and MgO in the indirect estimation.

† CaO and MgO were not estimated in the individual apples, but on a mixed sample of the same apples.

‡ Percentage calculated with reference to the amount found by analysis.

Table III gives the results of 30 analyses of individual apples; further details of these analyses have already been published [Brown, 1927]. Potash was estimated by analysis of the whole apple, but as is shown later the potash

contents of the freshly extracted juice and of the whole apple agree closely, probably within the limits of error. The "equivalent potash" estimated by the indirect method in the juice includes, as explained above, the "potash equivalent" of the lime and magnesia present, which were not estimated directly in the individual apples, owing to the small amount of material in each apple. For this reason the lime and magnesia percentages have been included in the comparison of means, the values being taken from the analysis of a mixed sample of 30 of the same apples. When the two sets of estimations are thus made comparable they agree very closely, the difference being little more than 6 %; moreover, the lime and magnesia percentages were obtained from the analysis of the whole apple and not of the juice and are therefore probably too low, for, as will be shown in the sequel, the mineral analyses of the juice and the whole apple are not the same. Where the "equivalent potash" by analysis is higher than that obtained by the indirect method this is probably to be ascribed to the presence of mineral acids, particularly sulphuric, as stated above (p. 952), but sulphate was not estimated in any of the analyses quoted owing to the special methods of ashing required. However, some samples of juice were analysed for sulphate in the early stages of this work, using the ordinary method of ashing which, according to Stockholm and Koch [1923], gives low results. In the juice of Bramley's Seedling apples from different localities 7-15 % of sulphate was found in the ash, and in a sample of the juice of Lane's Prince Albert apples 10 % of sulphate was found. It has already been pointed out that the effect of the addition of small amounts of sulphate to a buffer mixture of potassium malate and malic acid is to immobilise an equivalent amount of potash (or lime or magnesia) so far as  $p_H$  determinations are concerned, and therefore to cause the value of the "equivalent potash" calculated from  $p_H$  values to be lower than that found by mineral analysis of the juice. The correction to be added to the calculated value of the "equivalent potash" would therefore be of the order of 0.02 g. when the value of sulphate is of the order of 10 % of the total ash.

(3) *Titration and  $p_H$  of the juice of various apples and their "equivalent potash."*

During the course of this work a large number of determinations of  $p_H$  have been made on various kinds of apples, and the results are shown in Table IV. There were 10 apples in each sample and they were analysed shortly after picking. More recent experience has shown that larger samples would have been better, as samples of 10 show rather wide variations. However, it appears from the results that the salt content of apples does not vary greatly, but in Cox's Orange Pippin apples it is considerably higher than in other apples. These apples have also an unusual capacity for storing sugar and the two phenomena may be connected. Apart from this fact there is little in the results of Table IV to suggest that the percentage of "equivalent potash" in apples is determined by the variety.

Table IV. *Titration,  $p_H$  and equivalent potash of various apples.*

Variety	Locality, etc.	Titration (mg. equiv. per litre)	$p_H$	"Equivalent $K_2O$ " (g. per 100 cc.)
Bramley's Seedling	Lincolnshire	192.0	2.90	0.141
	Near Cambridge	173.0	3.03	0.174
	Near Bristol	130.5	3.05	0.127
Lane's Prince Albert	Near Bristol.	1st picking	150.0	2.96
		2nd picking	115.0	3.05
		3rd picking	96.0	3.18
Cox's Orange Pippin	Near Bristol.	1st picking	81.0	3.51
		2nd picking	82.0	3.62
		3rd picking	63.5	3.64
Allington Pippin	Near Bristol	134.5	5.09	0.147
Lord Derby	Near Bristol.	Young trees	58.0	3.35
		Trees of medium age	60.0	3.48
		Old trees	60.0	3.47
		Cultivated soil	62.5	3.36
		Uncultivated soil	44.0	3.64
				0.161
Dymock Red	Near Bristol	40.0	4.03	0.174
Sweet Alford	Near Bristol	17.5	4.16	—

Table V. *Titration,  $p_H$  and "equivalent potash" of apples in store.*I. *Lane's Prince Albert apples, 1921-2.*

Date	Titration (mg. equiv. per litre)	$p_H$	"Equiv. $K_2O$ " (g. in 100 cc.)	Titration (mg. equiv. per litre)	$p_H$	"Equiv. $K_2O$ " (g. in 100 cc.)
Sept. 26 before storage	115.0	3.05	0.118	—	—	—
Oct. 13	104.0	3.14	0.127	94.0	3.18	0.118
Nov. 8	107.5	3.08	0.113	81.0	3.27	0.122
Dec. 8	94.0	3.16	0.118	77.0	3.29	0.122
Jan. 11	72.0	3.25	0.104	52.0	3.67	0.141
Jan. 24	85.0	3.25	0.122	61.0	3.44	0.118
Feb. 9	—	—	—	56.5	3.48	0.118
Feb. 28	71.5	3.31	0.113	55.0	3.54	0.122
Mar. 14	75.0	3.33	0.122	45.5	3.68	0.127
Mar. 26	77.0	3.30	0.122	37.5	3.78	0.113
Apr. 4	92.5	3.24	0.132	45.5	3.61	0.113
Apr. 20	76.0	3.29	0.118	42.5	3.67	0.113
May 17	—	—	—	39.0	3.68	0.104
Mean $K_2O$	—	—	0.1191	—	—	0.1192

II. *Cox's Orange Pippin, 1921-2.*

Sept. 26 before storage	82.0					
Oct. 13	78.0	3.60	0.198	35.5	4.05	0.156
Nov. 10	66.5	3.64	0.179	44.5	3.90	0.165
Dec. 8	59.0	3.74	0.179	43.5	4.03	0.188
Dec. 22	—	—	—	36.5	4.19	0.188
Jan. 11	53.5	3.85	0.188	39.5	4.19	0.212
Jan. 23	38.0	4.25	0.188	—	—	—
Feb. 9	38.5	4.13	0.188	—	—	—
Mar. 2	39.5	4.02	0.170	—	—	—
Mar. 14	36.5	4.14	0.179	—	—	—
Mar. 24	40.5	4.09	0.188	—	—	—
Apr. 4	33.5	4.30	0.203	—	—	—
Apr. 20	47.0	4.00	0.198	—	—	—
Mean $K_2O$	—	—	0.1871	—	—	0.1818

In order to find out whether any sudden change of  $p_H$  occurred during storage, measurements were made at short intervals on samples withdrawn from store (Table V). The change in  $p_H$  was found to vary approximately with the titration, but the "equivalent potash," which would be expected to show some decrease even at low temperatures, owing to loss of water from the apple, was found to remain almost constant, probably owing to the decreasing acidity of the juice, for, as is shown in the sequel, magnesium is extracted from the cell wall during the expression of the juice and the amount extracted decreases with acidity. The increasing proportion of phosphoric acid in the juice also tends to make the value of the "equivalent potash" rather low, and is almost certainly the cause of the abnormally low results obtained occasionally in the last sample of the season when acidity has fallen very low. (No striking example of this is shown in the tables but it has been observed in samples where the acidity of acid apples has become much reduced.) It thus appears that comparisons of "equivalent potash" should be made as far as possible in similar conditions of acidity. Table IV shows the order of variations of "equivalent potash" in successive samples. The comparison of the three sets of pickings of Lane's Prince Albert and Cox's Orange Pippin (Table IV) is of interest: the results afford evidence that the first picking of Lane's and the first two pickings of Cox's are definitely higher in salt content than those which follow. This may be a consequence of the intake of water from rain and atmospheric moisture after the salt supply from the root has ceased, or it may mean that the apple is drawing upon a store of salts which is becoming depleted. The salt content of the apple is probably a factor of some importance in relation to keeping properties and although little evidence can be adduced in support of this statement one observation of some interest may be quoted. A set of Lord Derby apples from young trees which were put into store succumbed so completely to physiological breakdown after a few weeks that only a few survivors were left; these survivors were, however, in good condition. In the original sample before storing, estimated on October 20th, the percentage of soluble salt calculated in terms of potash, as shown in Table IV, was only 0.134, but for the survivors, estimated on November 15th, it was found to be 0.188. It will be noticed (Table IV) that the original sample of the apples from the young trees contained very much less salt than a sample from older trees, but the survivors of the apples from the young trees contained about the same amount. Definite conclusions cannot of course be drawn from a single series of observations, but the facts are highly suggestive, and it may well be that the bad keeping properties of apples from young trees, which is generally recognised, is due to an intake of water in excess of that of salts.



(4) *A comparison of the mineral content of whole apples with that of their juice and the changes in the composition of the juice during a period of storage.*

In order to reduce as far as possible the errors due to individual variation, samples composed of the different halves of the same apples were compared. Table VI, which gives the results of a preliminary investigation in which the properties of the juice from the two halves of a number of Bramley's Seedling apples were compared, shows that the mean differences between samples of the two halves of apples is very small, especially if the apples are cut longitudinally.

Table VI. *Density,  $p_H$  and acidity of the two halves of Bramley's Seedling apples.*

Apples cut longitudinally.							
Apple	Density of juice		$p_H$		Acidity (mg. equiv. per litre)		
1	1.051	1.051	3.11	3.15	108.5	107.0	
2	1.045	1.045	3.20	3.21	104.5	97.5	
3	—	—	3.50	3.52	73.0	67.0	
4	1.053	1.051	3.15	3.13	127.0	121.0	
5	1.050	1.050	3.40	3.30	109.0	115.5	
6	1.048	1.049	3.30	3.23	95.5	97.0	
7	1.053	1.055	3.15	3.11	125.0	129.0	
8	1.053	1.054	3.21	3.21	138.0	140.0	
Mean values	1.050	1.051	3.25	3.23	110.0	109.4	
Mean difference between two halves	0.001		0.04		0.43		
Mean deviation from mean	0.0025	0.0023	0.11	0.09	1.4	1.7	
Apples cut transversely: (a) = stalk end; (b) = calyx end.							
	(a)	(b)	(a)	(b)	(a)	(b)	
1	1.049	1.051	—	3.38	112.0	113.0	
2	1.047	1.051	3.22	3.26	100.0	112.0	
3	1.052	1.053	3.74	3.44	82.0	101.0	
4	1.046	1.050	3.21	3.15	110.5	120.0	
5	1.045	1.045	3.21	3.33	108.5	98.0	
6	1.048	1.052	3.24	3.10	117.0	128.0	
7	1.050	1.052	3.18	3.25	94.0	90.0	
8	1.048	1.049	3.18	3.16	100.5	100.5	
9	1.046	1.050	3.25	3.09	93.0	97.5	
Mean values	1.048	1.050	3.28	3.27	102.0	106.6	
Mean difference between two halves	0.003		0.11		0.89		
Mean deviation from mean	0.0017	0.0017	0.12	0.11	0.9	1.0	

Thirty Bramley's Seedling apples were used for the experiment described below. They had been in cold store at 1°, from the time of gathering until February 1st, when the experiment was begun. The apples were weighed in bulk and cut in halves, longitudinally as symmetrically as possible. One half of each apple was used at once for analysis, while the other half was stored at room temperature. The halves stored were kept on clock glasses with the cut surface immersed in paraffin wax. These were analysed in two batches of 15 apples each, one after a month and the other after 6 weeks' storage.

Precautions<sup>1</sup> to avoid fungal infection were taken during the cutting of the apple. Each apple was washed with a wad of cotton wool dipped in alcohol, and the cutting knife of stainless steel was sterilised by dipping in alcohol and burning before the cutting of each apple. The half of the apple to be stored, with the cut half held always downwards, was placed immediately upon a clock glass containing clean melted paraffin and previously sterilised by pouring on to it a little alcohol which was then set on fire. The stored half-apples thus treated kept as well as whole uncut apples. Out of the 30 only two suffered badly from fungal attack; both of these were in the first (random) sample analysed after 1 month's storage. The three lots of half-apples, 30 half-apples at the beginning of the experiment, 15 of the other halves after 1 month's storage and those remaining after another fortnight's storage, were all treated exactly similarly. The apples were peeled, cored, cut up and thoroughly mixed and any "bad" portion was removed. About 200 g. were weighed into platinum dishes for the estimation of total ash, potash, phosphate and magnesium in the fresh apple. The remainder was frozen and the juice expressed in the ordinary way except that boiling-tubes, covered on the outside with tinfoil, and a porcelain press were used in the place of metal vessels in order to avoid metallic contamination. In addition to the above-mentioned comparisons the juice of each of the two lots of 15 half-apples was expressed in two fractions in order to test whether the mineral matter of the runnings varied, but the second running gave very little juice, and this was not sufficient for a complete analysis. The acidity, density, and sugar content of the juice was determined immediately, and portions of the juice were measured out into platinum dishes for the estimation of total ash, potash, phosphate and magnesia. The remaining juice was heated to 70° to destroy pectinase and other enzymes, and bottled with thymol as preservative, so that the  $p_H$  of the juice of all the samples could be determined. The samples of juice and apple for mineral analysis were incinerated slowly in an electric muffle furnace, which was gradually allowed to reach a temperature of about 400°. The details of analysis were those previously described by one of the authors [Brown, 1926].

One sample of Dabinett apples was also analysed; this is a bitter-sweet variety grown for cider. The total ash, potash, phosphate and magnesia were estimated on the juice and the whole apple as before. For these apples the "equivalent potash" in the juice calculated from the  $p_H$  and titration values was very much lower (about 35 %) than that actually found by analysis. However, since the juice coagulated on heating, owing to its low acidity, before the destruction of pectinase, the  $p_H$  values were probably not dependable, and therefore the  $p_H$  and calculated "equivalent potash" have been omitted from Table VII; it may also be possible that in these apples with peculiar properties and low acidity the malate comparison is invalid.

<sup>1</sup> The authors desire to express their acknowledgement to Dr A. S. Horne for advice as to these precautions.

Sugar was determined on the samples of juice using Lane and Eynon's [1923] methylene blue method [Evans, 1928], and the acid by titration to a standard colour with phenolphthalein; the percentages were found from the weights in 100 cc. of juice by multiplying by the factor  $\frac{100-z}{100\sigma}$ , where  $z$  is the residue left from alcoholic extraction (taken as cell wall material) and  $\sigma$  is the specific gravity of the juice.

The results of these analyses in terms of comparable quantities are shown in Table VII. It is at once clear that the ash content of the juice is markedly higher than that of the whole apple. This is not due to any deficiency of ash in the cell wall, for this, which is normally less than 2 % of the whole, has been found after exhaustive extraction with alcohol to contain approximately the same percentage of ash as the apple. Most of this difference is undoubtedly due to contamination of the juice, for the silica contents of the samples of ash were determined, and it was found that the percentage of silica in the juice was about 15 times as great as in the whole apple. Thus the juice must have been contaminated by small chips of silica from the rough surface of the porcelain press, which was an old one. The figures for silica-free ash show such differences (within the limits of probable error) between the ash of the whole apple and the juice as might be expected if some solution of cell wall mineral matter had taken place, and it will be shown that there is much reason to believe that this has occurred. According to Loew [1899] the magnesia of plants is chiefly present in the cell wall in close association with lecithin, and he considers that magnesium is related to the assimilation of phosphates for the formation of lecithin and nucleoproteins. It is probable that calcium also may be present in the cell wall, most probably as a compound with pectin or pectic acid. When the juice is pressed out it passes through the cell walls, and it is obvious that some proportion of the mineral substances present there may be washed out, particularly when the juice is acid. If all the 98 g. of juice present in 100 g. of apple could be obtained by pressing, it should be expected that the total ash content of the juice and the whole apple would be the same, but only about 70 % of juice is obtained when killed apple tissue is pressed in the ordinary way; about 140 g. of fresh material are therefore required to give 100 g. of juice and if this juice contains material dissolved from the cell walls it may be derived from those of that weight of fresh apple. This would cause the ash content of the juice to be slightly higher than that of the whole apple.

It is interesting to see whether the figures for the individual constituents accord with this theory. It was not expected that any of the potash would be derived from the cell wall, and the figures in Table VII support this, for the differences between the percentages of potash in the juice and the whole apple are very small, and are within the limits of error. It thus seems likely that all, or nearly all, the potash of the apple is present in the cell sap. In this connection it may be mentioned that one analysis of the alcohol-insoluble residue

**Table VII.** *A comparison between the composition of apples and their juice.*

**I. Bramley's Seedling apples at intervals during storage.**

Sample and date	Mean weight of half-apples (g.)	% of juice from 100 g. apple	I. Total ash					II. Phosphate (P <sub>2</sub> O <sub>5</sub> )				III. Magnesia (MgO)			
			Apple		Juice		SiO <sub>2</sub> -free ash	Apple		Juice		Apple		Juice	
			A	SiO <sub>2</sub>	A	SiO <sub>2</sub>		A	B	A	B	A	B	A	B
Original sample Feb. 1 (30 halves)	70	—	0.192	—	0.260	0.214	0.0208	10.8	0.0188	0.0084	2.80	0.0054	2.62	0.0060	
Mar. 1* (15 halves) 1st pressing of juice	66	62	0.179	0.0005	0.220	0.0050	0.0204	11.4	0.0162	0.0047	2.62	0.0047	2.62	0.0060	
2nd pressing of juice	20	20	0.269	—	0.215	0.204	—	—	—	—	—	—	—	0.0059	
Mar. 15* (15 halves) 1st pressing of juice	69	52	0.190	0.0005	0.220	0.0055	0.0213	11.2	0.174	0.0053	2.79	0.0053	2.79	—	
2nd pressing of juice	17	17	0.185	0.0005	0.196	0.190	0.0208	11.3	0.0168	0.0050	2.72	0.0050	2.72	0.0060	
Mean of the two stored samples	68														
II. <i>Dabinett apples.</i>															
Feb. 16 (40 apples) 1st pressing of juice	35	35	0.338	0.0028	0.302	0.048	0.0214	6.35	0.186	0.0070	2.07	0.0070	2.07	—	
2nd pressing of juice	25	25	SiO <sub>2</sub> free - 0.335	—	0.359	0.044	0.315	—	—	—	—	—	—	—	

## II. *Dabinett apples.*

Sample and date	IV. Potash ( $K_2O$ )				H ion conc. $\times 10^{-2}$	$pH$	V. Properties of the juice			"Equivalent $K_2O$ "			
	Apple		Juice A	Acidity (mg. per litre)			Specific gravity	Sugar		Total .1	Direct estimation by analysis $K_2O$ and $MgO$ calc. as equiv. $K_2O$ †		Indirect estimation from $pH$ and titration values A
	A	B						Reducing A	Total .1		Apple A	Juice A	
Original sample Feb. 1 (30 halves)	0.106	55.4	0.107	0.1122	2.95	1.046	8.43	10.2	0.119	0.127	0.120	0.130	—
Mar. 1* (15 halves) 1st pressing of juice	0.0995	55.6	0.100	0.0977	3.01	1.043	8.75	9.98	0.110	0.114	0.110	0.110	—
2nd pressing of juice				—	—	1.043	—	—	—	—	—	—	—
Mar. 15* (15 halves) 1st pressing of juice	0.110	57.8	0.109	0.1122	2.95	1.037	—	10.20	0.123	0.123	0.123	0.100	—
2nd pressing of juice				0.1047	2.98	1.039	—	10.09				0.110	
Mean of the two stored samples	0.105	56.2	0.104	0.104	2.98	1.040	—	10.09	0.117	0.118	0.117	0.106	
Feb. 15 (40 apples) 1st pressing of juice	0.183	54.3	0.180	0.0813	25.0	1.064	13.8	16.34					
2nd pressing of juice			0.181	0.0692	25.0	1.064	13.8	15.33					

The percentages for analyses after 4 and 6 weeks' storage are referred to fresh weight at time of analysis, and not to original fresh weight, as the loss of weight is small.  
For 4 weeks' loss = 3.5% approx.; for 6 weeks' loss = 2.0% approx.; for 8 weeks' loss = 1.5% approx. (figures taken from another sample of the same apple).  
† CaO was not estimated.

The percentages for analyses after 4 and 6 weeks' storage are referred to fresh weight at time of analysis, and not to original fresh weight, as the loss of weight is small.

+ Ca() was not estimated.

of the apple was made, and the residue from 100 g. of apple contained 0.0011 g. of potash; thus it is possible that a very small percentage of the potash, about 1 %, is present in the cell wall. This percentage may be even less, for, since potassium sulphate is insoluble in alcohol, some potassium sulphate originally in the sap may have been held in the residue.

It is difficult to discover whether any cell wall phosphorus could be dissolved by the juice, since the phosphorus in the form of lecithin would not be expressed by the juice, and this large difference between the amounts of phosphorus in the juice and the whole apple would mask any small difference that might occur owing to the solution of a small percentage of phosphate that might be present in the cell wall. It is probable, however, that most of the free phosphates of the apple as well as any hexosephosphate that may occur are present as potassium salts in the cell sap, and that the cell wall contains little more than the phosphate which is combined as lecithin. This conclusion is supported by the figures of Table VII, which show that the difference between the amount of phosphorus in the juice and in the whole apple is from 10 to 20 %, for this percentage of phosphorus as lecithin was previously found [Brown, 1927] in a preliminary determination of phosphorus compounds in apples. In the Dabinett apples, however, the second running of juice contains less phosphate than the first; this suggests that the first running may have dissolved some phosphate from the cell wall, but this single result needs confirmation.

The figures for magnesia, however, afford much support to the theory of the solution of cell wall material by the juice, for the magnesia content of the juice is from 15 to 40 % higher than that of the whole apple. The maximum figure is that which would be obtained if none of the magnesia remained in the cell wall, for the weight found in 100 g. of juice corresponds with that from 140 g. of fresh material. The calcium was not estimated, but in the process of determining magnesia, calcium is first precipitated and it was noted that there was always a larger precipitate of calcium from the ash of the juice than from that of the whole apple. In some earlier work on Lane's Prince Albert apples from East Malling, given in Table II, it will be seen that a much larger percentage of lime was present in the juice than in the whole apple, which supports the evidence derived from the magnesia figures. It might be expected that the first runnings of juice would contain more mineral matter derived from the cell wall than the second running, and the figures of Table VII support this, although the differences are too small to be entirely conclusive.

It is interesting to observe the changes which took place during the 6 weeks' storage. The differences obtained in the samples stored for 4 and 6 weeks respectively must be almost entirely due to sampling, for the changes during a fortnight would hardly be detectable, and it is obvious that the small increase of acidity during the last two weeks of storage is due to individual variation. The mean of the two analyses after 4 and 6 weeks should therefore give an accurate measure of the state of the apple after 5 weeks' storage and

it is interesting to note that, although the properties of the juice have altered, the percentage of mineral constituents in the fresh apple remains unchanged, showing that the sampling error has been reduced by using half-apples. The changes in the composition of the juice are considerable, acidity and specific gravity have fallen and  $p_H$  has increased correspondingly; these are normal effects of storage. On the other hand, it is to be noted that the percentages of phosphate and magnesia in the juice are less, and this agrees well with what has already been deduced as to the solution of the mineral constituents of the cell wall by juices containing acid.

#### SUMMARY.

1. A method of calculating the value of "equivalent potash" content of apple juice from titration and  $p_H$  values is suggested.

2. Measurements of titration,  $p_H$  and "equivalent potash" are given for samples of Lane's Prince Albert and Cox's Orange Pippin picked at the normal season and also earlier and later. Similar measurements on samples from a single picking of other kinds of apple are also given.

3. A comparison has been made between the mineral content of apples and that of their expressed juice on two varieties of apples.

The same percentage of potash was found in the expressed juice as in the whole apple, indicating that most of the potash of the apple is present in the cell sap. Larger percentages of magnesia and lime were found in the juice than in the whole apple, from which it is concluded that the juice has dissolved lime and magnesia from the cell wall. There is some evidence that this solution of cell wall material by the juice varies with the acidity of the juice.

#### REFERENCES.

- Brown (1926). *Ann. Bot.* **40**, 129.  
— (1927). *Ann. Bot.* **41**, 127.  
Evans (1928). *Ann. Bot.* **42**, 1.  
Franzen and Helwert (1923). *Z. physiol. Chem.* **127**, 14.  
Haynes (1921). *Biochem. J.* **15**, 440.  
— (1925). *Ann. Bot.* **79**, 77.  
Haynes and Judd (1919). *Biochem. J.* **13**, 272.  
Lane and Eynon (1923). *J. Soc. Chem. Ind.* **42**, 32 T.  
Loew (1899). *U.S. Dept. Agric. Div. Veg. Physiol. Pathol. Bull.* no. 18.  
Nelson (1927). *J. Biol. Chem.* **40**, 1300.  
Stockholm and Koch (1923). *J. Amer. Chem. Soc.* **14**, 1953.

## CXX. THE GLUCOSE METABOLISM OF KIDNEY TISSUE *IN VITRO*. II.

BY JAMES TUTIN IRVING (*Beit Memorial Research Fellow*).

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(*Received June 30th, 1928.*)

IN a previous communication [Irving, 1927] it was shown that the disappearance of glucose from phosphate buffer in which chopped kidney cortex was suspended was markedly inhibited or completely stopped when the incubation was carried out under anaerobic conditions or in the presence of small amounts of HCN. In this paper, experiments are recorded which deal with the fate of the glucose utilised.

### EXPERIMENTAL.

The technique employed was in all respects the same as that previously described. The kidneys were perfused till free from blood, and the cortex was cut off and chopped with a razor into small cubes which were well shuffled. In most experiments 2 g. were placed in 8 cc. of phosphate buffer,  $p_H$  8.2. This suspension was then treated as described below. The tubes, which were incubated at 37.5°, were continually shaken by a mechanical device. The precautions as regards sterility were as described in the earlier paper. The glucose added was Kahlbaum's purest, and the sugar estimations were carried out by Hagedorn and Jensen's method. Incubation in no case exceeded 2 hours.

The writer had suggested previously that the glucose utilised by kidney tissue might be going, in part at least, to form polysaccharides. Further work has shown that this does not take place. Hydrolysis of the buffer solution in which the kidney cortex had been suspended after incubation with and without glucose showed small and equal rises in reducing power in both cases. Glycogen estimations undertaken on the kidney tissue before and after incubation showed that this substance fell in amount, and synthesis did not take place. The method of estimation used was shown to give quantitative figures on pure glycogen and also on glycogen added to kidney tissue. Typical figures are shown in Table I.

Table I.

2 g. kidney cortex. 8 cc. phosphate buffer. . 8 mg. glucose. 2 hours' incubation at 37.5°.		
	Glycogen content (mg.)	
Glucose utilised (mg.)	Before	After incubation
5.52	1.36	1.06
10.0	1.91	0.96
None added	1.28	0.87

Lactic acid estimations were also undertaken. Clausen's method [1922] and that recently described by Friedmann, Cotonio and Shaffer [1927] were employed. The claims made for the latter method by the authors were found to be fully substantiated. Whereas Clausen's method gave only 85 % of the value on pure lactic acid, the newer estimation gave 97 to 100 % recovery.

After the necessary glucose samples had been taken, the entire suspension was cooled in ice and ground in an ice-cold mortar with cold tungstic or trichloroacetic acid. It was then made up to a known volume and centrifuged. An aliquot part of the supernatant fluid was saturated with ammonium sulphate and acidified with phosphoric or sulphuric acid. The lactic acid was then extracted with specially purified ether, either by using a continuous extractor as devised by Clausen or by shaking five times with three volumes of ether as described by Fletcher and Hopkins in their original paper. This method was found to give theoretical figures with pure lactic acid solutions, but slightly (about 8 %) less with kidney tissue. The necessary corrections have been made in all the figures quoted. The ether gave no blank value.

In the presence of glucose either ether extraction or the copper-lime procedure must be adopted. In the course of some work undertaken by Dr H. D. Kay and the writer, it was found, however, that protein-free kidney filtrates contain some substance which reduces permanganate very strongly, and which thus interferes with the estimations. This substance is still present after treatment with the copper-lime reagents, but is not soluble in ether. The ether extraction method is thus the only one applicable to the kidney.

The results obtained on the kidney suspension incubated with, and without, glucose were that the bulk of the sugar utilised was converted into lactic acid, positive thiophen tests showing that the figures obtained were not due to some substance simulating lactic acid. In order to confirm the results previously obtained, pairs of experiments were undertaken simultaneously on the same kidneys, one sample being aerobic, the other either anaerobic or in presence of HCN. In the case of the second sample, the glucose utilisation and the lactic acid production were always found to be greatly diminished. Typical results are given in Table II. In the cases shown, allowance has been made for the production of lactic acid from its precursors in the kidney tissue. In most cases it was found that the lactic acid maximum had been reached before incubation was begun. HCN, in the concentration present in the final estimation, was found to have no effect upon the lactic acid values.

It will be seen that the percentage transfer of glucose to lactic acid in each pair of samples was fairly constant, though it differed widely from experiment to experiment. This would suggest that the same processes were taking place aerobically, and to a less extent, anaerobically. The fact that with more rapidly acting kidneys the transfer was lower might be evidence that the lactic acid formed was being further oxidised, as shown by Meyerhof and Lohmann [1926], but experiments carried out to test this have given no decisive answer. The main conclusion which can be reached from these figures is that the formation of lactic acid from glucose was markedly inhibited under anaerobic conditions.



Table II.

2 g. kidney cortex.  
8 cc. phosphate buffer.  
10-15 mg. glucose.  
2 hours' incubation at 37.5°.

Exp. no.	Control sample			Experimental sample			Conditions
	Glucose utilised (mg.)	Lactic acid formed, net. (mg.)	% formation of lactic acid	Glucose utilised (mg.)	Lactic acid formed, net. (mg.)	% formation of lactic acid	
1	13.88	7.01	51	8.18	5.33	65	Anaerobic
2	7.76	5.99	77	2.64	1.82	69	"
3	5.93	5.79	98	3.08	2.57	84	"
4	12.08	7.63	63	3.90	2.03	52	HCN M/500
5	7.95	2.06	26	2.78	0.99	36	"
6	4.24	3.79	90	1.84	1.88	102	"

Simpson and Macleod [1927] have found that under certain conditions the lactic acid produced in cat muscle is lower in amount than the glycogen disappearing, thus indicating the production of intermediate substances. Meyerhof [1927] has shown that during the degradation of carbohydrates by the muscle enzyme a relatively stable phosphoric ester accumulates, which he considers is hexosediphosphoric acid. On analogy with this, the possibility existed that in this case the excess glucose had become esterified, and experiments were undertaken to test this.

Owing to the small amount of tissue available, phosphorus estimations at the end of 2-hour periods only have so far been attempted. The kidney cortex was incubated in isotonic sodium carbonate solution at a  $p_H$  of about 8.3 in two tubes, one with, and one without, glucose. Samples for glucose estimations were taken and the whole suspension was then extracted as usual with 4 % trichloroacetic acid and centrifuged. Free phosphorus determinations were then made directly on one portion of the supernatant fluid by Briggs's method, and another portion was extracted for lactic acid determinations.

In all the experiments, a slight fall in the inorganic phosphorus content was found in the tube which had contained glucose, as compared with the control tube. In some cases the fall was sufficient to account for all the glucose not turned to lactic acid if it was assumed that hexosediphosphoric acid was formed, while in some there was still glucose not accounted for. This might be due to the formation of a mixture of mono- and di-phosphoric esters. Typical figures are given below.

Table III.

2 g. chopped kidney cortex.  
8 cc. isotonic  $\text{Na}_2\text{CO}_3$  solution ( $p_H=8.3$ ).  
8-10 mg. glucose.  
2 hours' incubation at 37.5°.

Exp. no.	...	1	2	3
Glucose utilised (mg.)	...	4.35	5.78	2.78
Lactic acid produced (mg.)	...	3.82	4.85	1.74
Glucose unaccounted for (mg.)	...	0.53	0.93	1.04
Fall in free phosphorus (mg.)	...	0.19	0.22	0.11
Phosphorus required for hexosediphosphoric acid (mg.)	...	0.18	0.27	0.36

It was also observed that the rate of utilisation of glucose in carbonate solution was as a rule slower than in phosphate buffer. It is thus very probable that organic phosphorus compounds act as intermediaries in this reaction.

The fact that this mechanism in the kidney differs so markedly from that in muscle is no doubt bound up with its special function as a secreting organ. The writer has as yet no evidence as to the part which this metabolic reaction plays in that function, but, as he is unable temporarily to investigate the subject further, it has appeared advisable to report the progress made.

#### SUMMARY.

Glucose is changed in large part to lactic acid by kidney tissue *in vitro*. A small amount is esterified with phosphoric acid. The utilisation of glucose and the production of lactic acid are strongly inhibited *in vacuo* or in the presence of HCN.

The writer is indebted to Prof. R. A. Peters for his continued advice and interest in this work.

The expenses of this research were defrayed by a grant from the Government Grant Committee of the Royal Society.

#### REFERENCES.

- Clausen (1922). *J. Biol. Chem.* **52**, 263.  
Friedmann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.  
Irving (1927). *Biochem. J.* **21**, 880.  
Meyerhof (1927). *Biochem. Z.* **183**, 176.  
Meyerhof and Lohmann (1926). *Biochem. Z.* **171**, 381.  
Simpson and Macleod (1927). *J. Physiol.* **64**, 255.

## CXXI. TYRAMINE OXIDASE.

### I. A NEW ENZYME SYSTEM IN LIVER.

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(Received June 30th, 1928.)

THE properties of tyramine, *p*-hydroxyphenylethylamine, have hitherto been studied almost entirely from the physiological, rather than from the biochemical standpoint. Dale and Dixon [1909] have reported that its effects on injection are similar to those produced by adrenaline, though less well marked. It is known to be one of the products produced from protein by the action of certain bacteria in the intestine of the higher animals, and toxic conditions occur simultaneously with increased absorption of these products into the blood stream. Also, its chemical relationship to tyrosine, and to adrenaline and thyroxine, render of interest any contribution to our knowledge of its metabolism.

Ewins and Laidlaw [1910] investigated the effects of perfusing surviving organs with tyramine, and found that, in the case of the liver, the amine was transformed quantitatively into the corresponding *p*-hydroxyphenylacetic acid. They then attempted to isolate this acid from liver extracts after a period of incubation with tyramine, but failed to do so. Using the heart, they failed to find either tyramine or the acid after perfusion, and they believed that the benzene ring was broken down.

Work has also been done on tyramine in connection with the enzyme tyrosinase, which will utilise as its substrate aromatic compounds having a hydroxy-group in the *para*-position [Bertrand, 1907]. It attacks tyramine and produces a pigmented substance, probably a melanin.

In view of the paucity of studies on the catabolism of tyramine, and the improved methods now available for the detection of oxidative processes, it was decided to investigate the effect of the addition of tyramine on the oxygen uptake of various tissues. The Barcroft differential manometer was used, and it has been found that, with liver extracts, an oxidation of tyramine occurs. The oxygen uptake corresponds exactly to the absorption of one atom of oxygen per molecule of tyramine. Further study showed that this reaction involves not only oxidation, but also deamination, but that only half the maximum possible amount of nitrogen is evolved as ammonia. That is, one molecule of ammonia is formed from every two molecules of tyramine.

As will be shown in the experimental section, there are certain facts which clearly distinguish this system from tyrosinase. No pigment is produced, and tyrosine and *p*-cresol are not attacked. The addition of *M*/500 KCN has no effect upon the oxygen uptake, whereas tyrosinase is known to be extremely sensitive to cyanide [Lehmann, 1909]. It is therefore believed that this enzyme system has not hitherto been investigated. A preliminary account of its properties is given below, and further work is in progress.

#### EXPERIMENTAL.

*Preparation of the enzyme.* The livers of rat, rabbit, sheep, pig, ox and dog were found to be capable of oxidising tyramine, but that of the rabbit is especially active. Rabbit liver was therefore used in the experiments described below. The rabbit was killed by bleeding and the liver removed and finely minced. The minced liver was ground with washed sand, diluted with an equal volume of water and squeezed through muslin. The resulting liquid was very active and would keep for several days on ice, and for most experiments 1 cc. of it was used in each Barcroft bottle. This amount when fresh will oxidise 2 mg. of tyramine in about 3 hours at 15°, but it slowly loses its activity.

A cell-free solution of the enzyme was prepared by the addition of kaolin to the liver extract and adjusting to  $p_H$  6.5. The enzyme is adsorbed by the kaolin, which is centrifuged and washed. It is then removed from the kaolin by dilute soda ( $p_H$  8.0), and again centrifuged. The solution now contains the enzyme in a very active form, but it is unfortunately very unstable, and will not keep, even at 0°, for more than a few hours. No method has yet been found for preventing this destruction, which is probably due to a lack of the protective colloids present in the original extract. For certain of the following experiments minced liver was washed two or three times with distilled water, the liquid squeezed out through muslin, and the tissue, when required for use, was suspended in buffer or water. Difficulty was experienced in obtaining by this method a uniformly active preparation, as part of the enzyme is washed out into the water. Therefore the method of preparation first described was used for most experiments.

*Preparation of tyramine.* The process used was that of Johnson and Daschavsky [1925] as modified by Abderhalden and Gebelein [1926]. Briefly, tyrosine is heated to 240° in the presence of diphenylamine as a catalyst. Carbon dioxide is evolved, and the tyrosine gradually dissolves, until a clear liquid is obtained. After cooling, the tyramine may be separated from the diphenylamine by washing with ether, in which the former is almost insoluble. The crude tyramine may be converted into the hydrochloride, but was usually recrystallised directly from boiling xylene. The yield is very good, being about 90 % of the theoretical.

*Properties of the system.* The Barcroft apparatus was set up with 1 cc. of enzyme preparation and 1 cc. of buffer in each bottle, plus 1 cc. water in bottle (1), and 1 cc. tyramine solution in bottle (2). Phosphate buffer,  $p_H$  7.3,

was used unless otherwise stated. The tyramine solution was made up fresh each day, so that 0.5 or 1 cc. of the solution contained 2 or 3 mg. of tyramine. Control experiments were done without tyramine and also with tyramine alone.

The oxygen uptake is very rapid at the beginning of the reaction. For this reason, the apparatus was shaken in a bath at room temperature rather than at 37°, for the reaction is then slower and the errors consequently smaller. The whole reaction is complete in about 4 hours. Towards the end the oxygen uptake becomes slow and finally ceases, this being the typical curve to be expected from an enzyme system. The amount of oxygen absorbed under these conditions is from 85 to 90 % of the theoretical, if one molecule of tyramine

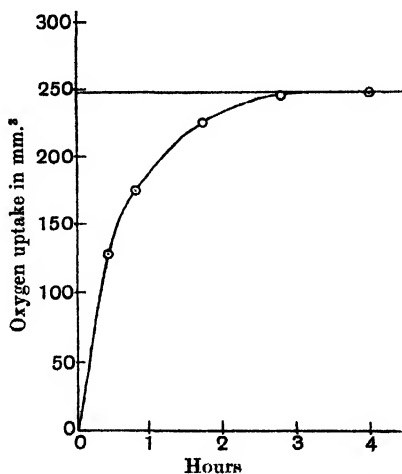


Fig. 1. The oxidation of tyramine by liver.

Theoretical oxygen uptake for 3 mg. tyramine - 245 mm.³

takes up one atom of oxygen. In these experiments, however, an error always occurs when the tyramine is added to the liver extract and the apparatus has to be shaken while open to the air to allow temperature equilibrium to be reached. This error was eliminated by the use of small jars in which the tyramine may be put inside the Barcroft bottles and then upset into the liver extract when the apparatus is closed. In this way, the oxygen uptake obtained corresponds exactly to the theoretical. An experiment is given. All figures are corrected to normal pressure and temperature for the dry gas.

The enzyme system is thermolabile, liver extract after boiling for 3 minutes showing no oxygen uptake whatever with tyramine. The system is unaffected by the presence of cyanide at a concentration of  $M/500$ , although the oxygen uptake of liver extract alone is approximately two-thirds inhibited. Other workers have shown that a concentration of  $M/1000$  KCN is sufficient completely to inhibit systems such as succinoxidase [Szent-Györgyi, 1924] and Warburg [1921, 1923, 1, 2] has put forward a theory that all oxidations

occurring in the cell are dependent upon the presence of traces of iron to "activate" the oxygen. Dixon [1925] has shown that the xanthine oxidase forms an exception to this hypothesis, and the tyramine oxidase is another example of the type of oxidation which is independent of traces of iron.

*Exp. 1.* Four Barcroft apparatus were used, and each bottle contained 1 cc. liver extract and 0.9 cc. buffer. Bottle (1) of each apparatus contained 0.5 cc. water, and bottle (2) contained 0.5 cc. (*i.e.* 2 mg.) tyramine solution, which was enclosed in a small jar. Apparatus *V* and *W* were controls, and contained 0.6 cc. water in both sides, whereas *X* and *Y* contained 0.6 cc. *M*/100 KCN instead. As no difference is obtained in the curves when cyanide is present, only isolated points are given. The figures (Table I) represent the oxygen uptake in mm.<sup>3</sup>

Table I.

Time (hours)	<i>V</i> (no KCN)	<i>W</i> (no KCN)	<i>X</i> (KCN <i>M</i> /500)	<i>Y</i> (KCN <i>M</i> /500)
0.5	107	99	111	113
1	149	138	142	148
2	161	155	160	160
3	164	162	167	163

The theoretical oxygen uptake for 2 mg. tyramine is 163 mm.<sup>3</sup>

Other organs were now prepared in the same way as the liver, and kidney, lung, suprarenals, skeletal muscle and heart were tested. Of these all were negative except the kidney, which showed a slight uptake with tyramine. It was expected that extract of heart would show an oxidation, for in the experiments of Ewins and Laidlaw the oxidation of the tyramine proceeded further with heart perfusions than with the liver. Several different preparations of heart were tried, but no oxidation could be obtained, so that it is evident that the mechanism of the oxidation is different in the case of the heart from that of the liver, in that in the former the system cannot be extracted and is probably dependent upon the intact organ structure.

Experiments were now carried out on the influence of different hydrogen ion concentrations on the activity of the liver tyramine oxidase. The liver extracts were taken to the required  $p_H$  by the addition of acid or alkali, and then suitably buffered, acetate, phosphate or borate buffer being used. Control experiments showed that the oxygen uptake of the tyramine was not influenced by any of these anions.

In all cases, a second portion of the extract was similarly treated, but was readjusted to  $p_H$  7.3 before adding the buffer, in order to show whether the system was actually destroyed or merely inhibited by the treatment. The activity was determined by the amount of oxygen taken up during the first hour. It will be seen that the enzyme is very much more active in alkaline solutions than in neutral, and reaches its optimum at  $p_H$  10.0, showing a sudden diminution in activity between  $p_H$  10.0 and 11.0. On the acid side, it is inhibited, but not destroyed, at  $p_H$  4.4 and it is completely destroyed in very alkaline solutions ( $p_H$  11.5). Control experiments were tried with tyramine alone, but no autoxidation was found to occur.

In view of the possibility that direct deamination might occur during this reaction, accompanying or independent of the oxidative process, ammonia estimations were done on the contents of the Barcroft bottles after the oxygen uptake had ceased. Such a deamination was found to occur, as the bottles with liver extract and tyramine contained very much more ammonia than the controls which contained liver extract and water. When washed liver or the kaolin preparation was used, the ammonia content of the controls was very small, but the content of fresh liver varied very much; nevertheless, the same amount of extra ammonia was present after the oxidation of the tyramine in every case. A vacuum distillation method was adopted for the estimation of the ammonia. The contents of the Barcroft bottle were washed out into the distilling flask, and 5 cc. 20 % sodium hydroxide were added. The ammonia was

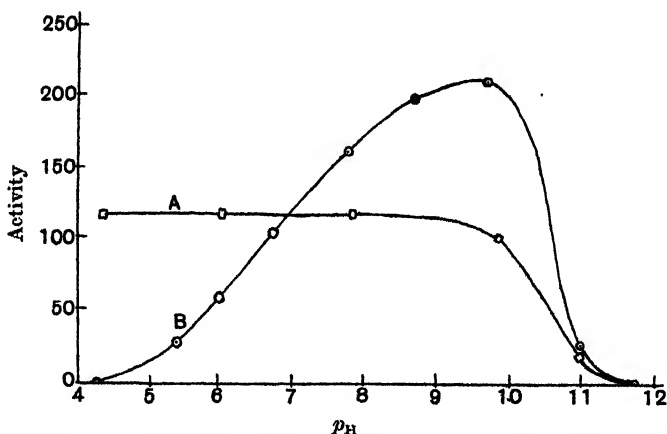


Fig. 2. The influence of hydrogen ion concentration on the activity of the tyramine oxidase system.

A—Activity of enzyme at  $p_H$  7.3 after being subjected for 5 mins. to various hydrogen ion concentrations.

B—Activity of enzyme at different hydrogen ion concentrations.

distilled into 3 cc.  $N/10$  HCl for 15 minutes at  $40^\circ$ . The distillate was diluted and nesslerised, one-tenth of the final volume of Nessler's reagent being used, and made up to 25 or 100 cc., the smaller volume being used for the washed liver or kaolin preparation controls, which contained very little ammonia. The colour of the nesslerised solution was compared with that of a standard in a colorimeter. No difficulty was experienced with the process of nesslerisation, and the resulting solutions were always crystal clear. Control experiments were done on tyramine alone, to show that it was not deaminised by distillation under these conditions.

It was found that the ammonia given off was only half that which is theoretically possible. This was at first thought to be due to a loss of ammonia from the Barcroft bottles, as the  $p_H$  of the reaction mixture, 7.3, was slightly on the alkaline side of neutrality. To eliminate this possibility a new apparatus was set up. Three small wide-necked bottles were tightly corked and con-

nected together with rubber and glass tubing, so that a stream of air could be sucked through them. The entry tube of each bottle reached to the bottom. The first bottle contained dilute sulphuric acid to wash the air, the second the buffered liver extract and tyramine, and the third *N*/10 HCl. The third bottle was connected to the pump, and air was slowly sucked through for about 5 hours, so that the oxidation of the tyramine should be complete. A control set of bottles was set up at the same time, in which the tyramine was replaced by water, and connected by a T-piece and taps to the same pump. The rate of the air current could thus be regulated so that it was the same for each set of bottles.

The ammonia distillations were done on the contents of the second bottle, and the liquid in the third bottle was washed out into the distillate before nesslerisation. The ammonia estimated was again found to be just half that which was expected, and it was demonstrated that the hydrochloric acid bottle contained only traces of ammonia at the end of the experiment. In view of the possibility that the presence of liver extract might alter the amount of ammonia that could be estimated, for instance by the conversion of some of the ammonia into urea, experiments were done in which a known amount of ammonium sulphate was added to the liver extract. After incubation, the ammonia was estimated as before, and the whole amount of the added ammonia was recovered. Ammonia estimations were also carried out on the heart extracts and tyramine after incubation, as it was thought possible that deamination might occur without oxidation, but the amount of ammonia was identical with that found in the controls. Table II shows these results.

Table II. *The deamination of tyramine by certain tissues.*

Tissue	Tyramine added (mg.)	Time of incubation in hours at 15°	Total NH <sub>3</sub> -N estimated (mg.)	NH <sub>3</sub> -N evolved from tyramine (mg.)
(1) 2 cc. liver extract	None	5	0.048	—
	3	5	0.195	0.147
(2) 2 cc. washed liver	None	6	0.011	—
	3	6	0.166	0.155
(3) 2 cc. washed liver	None	6	0.014	—
	3	6	0.172	0.158
(4) 2 cc. liver extract	None	5	0.130	—
	2	5	0.230	0.100
(5) 2 cc. kaolin preparation	None	5	0.044	—
	2	5	0.148	0.104
(6) 2 cc. kaolin preparation	None	5	0.046	—
	2	5	0.148	0.102
(7) 2 cc. heart extract	None	6	0.09	—
	3	6	0.08	None

*Control experiments.*

	NH <sub>3</sub> -N added as (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (mg.)		NH <sub>3</sub> -N recovered (mg.)
(8) 2 cc. liver extract	None	5	0.190
	0.100	5	0.295
(9) 2 cc. liver extract	None	5	0.195
	0.100	5	0.301



Liver extract was now incubated anaerobically in Thunberg tubes for several hours, 3 cc. buffered extract and 1 cc. tyramine solution or 1 cc. water were added and the tubes evacuated. After about 5 hours ammonia estimations were made and it was found that there was no increase of ammonia in those tubes which contained tyramine. This shows that the deamination is connected with the oxidative process and does not occur without the latter.

Further experiments were now carried out to show the relationship of the amount of ammonia produced at various stages to the oxygen absorption during the reaction. This relation is shown graphically in Fig. 3.

It will be seen that the deamination is proportional to the oxygen uptake, but that it ceases when only half the nitrogen of the tyramine molecule has been liberated as ammonia. When the oxidation is complete the deamination also stops, and no more ammonia is evolved.

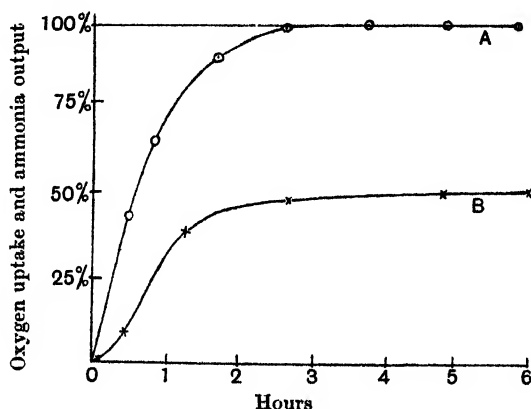


Fig. 3. Relationship of the deamination to the oxidation of tyramine by liver.

Values given as percentages and (a) the theoretical oxygen uptake as calculated for 1 atom per molecule of tyramine (A), and (b) the theoretical maximum ammonia nitrogen (B).

The Thunberg technique was now employed to see whether the presence of tyramine would accelerate the reduction of methylene blue by liver extracts. A great many experiments were carried out, but no considerable acceleration could be demonstrated with any of the liver preparations used. The explanation of this negative result may be that the reduction potential of the system is too low to reduce methylene blue, but that a dye having a higher  $r_H$  (that is a lower reduction potential) than methylene blue might be reducible. Therefore certain dyes (nos. 4, 6 and 9 in the Mansfield Clark series of  $r_H$  indicators, methylene blue being no. 3 a) were made up in solutions equivalent to methylene blue, 1/5000, and put up in vacuum tubes with liver extract and tyramine. The liver preparation was used, but dyes nos. 6 and 9 were reduced so rapidly by the extract alone that the experiment was not conclusive, and in the case of no. 4, no acceleration could be detected in the presence of tyramine. *m*-Dinitrobenzene was also tested as a possible hydrogen acceptor. The solid was finely ground, and about 0.2 g. was added to each Thunberg

tube. 2 cc. enzyme preparation, 2 cc. buffer, and 1 cc. tyramine solution or 1 cc. water were added and the tubes evacuated. After incubation for a few hours the tubes were opened and made alkaline by the addition of sodium hydroxide; reduction is indicated by the appearance of a dark red colour, but this was not deeper in the tyramine tubes than in the controls.

Experiments were now performed to see whether the production of hydrogen peroxide during the reaction could be demonstrated. The technique was that used by Thurlow [1925] and is based on the fact that hydrogen peroxide in presence of milk peroxidase oxidises nitrite to nitrate; the residual nitrite is estimated by acidifying and adding the Griess-Ilosvay reagent.

*Exp. 2.* Three flasks were set up, each containing 2 cc. liver extract in buffer,  $p_H$  7.3, plus 3 cc. milk peroxidase solution, and  $\text{NaNO}_2$  to make the final concentration  $N/1000$ ; to flask no. 1 was added 1 cc. water, to flask no. 2 1 cc. tyramine solution (3 mg.). Nothing was added to the third flask. The flasks were then incubated at room temperature for 5 hours. 1 cc. tyramine solution (3 mg.) was then added to flask no. 3, and immediately 2 cc. acetic acid and 2 cc. Griess-Ilosvay reagent were added to each flask.

Flask	Colour
1	+ + + + +
2	+ +
3	+ + + + +

Liver tissue alone reduces nitrate to nitrite [Bernheim and Dixon, 1928], so that a complete oxidation of the nitrite could not occur. Flask no. 3 in the experiment was put up to show that the diminution in the amount of nitrite was not due to the interaction of the amine group of the tyramine molecule and the nitrite after acidification. A concentrated solution of milk peroxidase was added to compete with the catalase present in the extract for the available hydrogen peroxide. The above experiment is typical of many.

Many other substances have been tried as possible substrates, including tyrosine, phenylalanine, dihydroxyphenylalanine, *p*-cresol, phenol, aniline and adrenaline, but these were found to be unattacked. *p*-Aminophenol is slowly autoxidisable at  $p_H$  7.3 and the addition of liver extract causes a slightly more rapid oxygen uptake. However no deamination occurs and it is not known whether the acceleration is due to the same system as the tyramine oxidation.

A few preliminary experiments have been performed with phenylethylamine. When this is neutralised and added to the Barcroft apparatus containing liver extract it shows an oxygen absorption equivalent to one atom per molecule, which is unaffected by the addition of  $M/500$  KCN. The production of hydrogen peroxide during the reaction can be demonstrated and marked deamination occurs. Thus it seems probable that the system responsible for this oxidation is identical with the tyramine oxidase; further work is in progress on this point.

The liver extract as used contains also other oxidising enzymes and certain

substances, such as *p*-phenylenediamine, are oxidised by it, but these are also oxidised by other tissues [Battelli and Stern, 1912], which have no effect on tyramine.

Certain substances were now added to the tyramine oxidising system, with a view to the possible inhibition of the reaction. The substances mentioned above, also ammonium sulphate, produced no effect. *p*-Hydroxyphenylacetic acid, the end-product in Ewins and Laidlaw's experiments, is also inactive in this respect. However, it was noticed that the further addition of tyramine to the reaction mixture in the Barcroft apparatus, after the original uptake had ceased, resulted in a renewed uptake which was slower than the first, but which eventually reached nearly the theoretical value. Further oxidation occurred after a third addition of tyramine, but the uptake was now greatly slowed (Fig. 4).

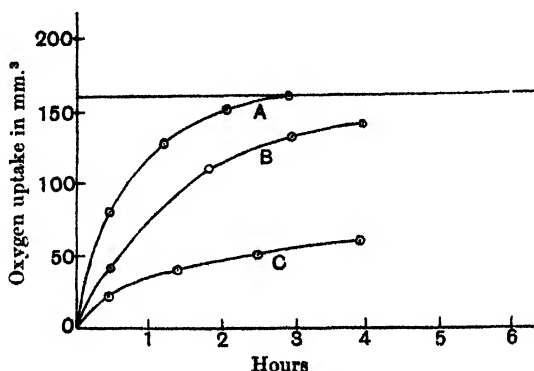


Fig. 4. The effect of successive additions of tyramine to liver extract.

Curves A, B and C represent respectively the oxygen uptake after the first, second and third additions of 2 mg. tyramine.

These facts suggest that the inhibition is due to an increasing concentration of the end-products of the reaction, and this is made still more probable by the following experiment. A mixture of liver extract and tyramine which has been incubated for several hours is deproteinised by the addition of "colloidal iron" and sodium sulphate, in the proportions suggested by Hiller and van Slyke [1922]. The clear filtrate is evaporated to dryness on a water-bath and a portion of the residue, about 10 mg., is added to both sides of a Barcroft apparatus, which contains liver extract and tyramine as usual. The result is a marked inhibition, as shown in the curve (Fig. 5).

Control experiments show that this effect is not due to an inhibition of the oxygen uptake of the liver preparation alone, and it is probably due to the increased concentration of the end-products.

The properties of the substance obtained as above by the removal of the protein from the reaction mixture were studied. An aldehyde seemed a possible product, though this, if formed, would probably undergo a Cannizzaro reaction. The presence of an aldehyde could not be demonstrated by any of the ordinary

chemical tests, and in view of the fact that the reaction does not involve complete deamination, the quantitative production of the corresponding aldehyde, or *p*-hydroxyphenylacetic acid, appears improbable. Further experiments are in progress on the amino-nitrogen content of the deproteinised residue. Large scale experiments are also in preparation, by means of which it is hoped to determine the nature of the final product of the reaction.

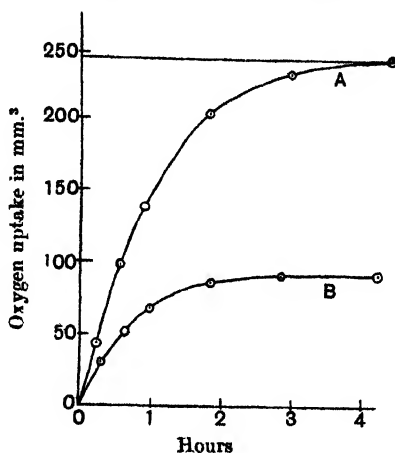


Fig. 5. The effect of addition of deproteinised residue on the oxygen uptake of the tyramine oxidase system.

A = control.

B = plus 10 mg. solid residue.

### DISCUSSION.

The oxidation of tyramine by liver, as discovered by Ewins and Laidlaw, which has been shown to be due to a definite enzyme system, may be of significance in three possible ways.

Firstly, the system may be protective and present for the purpose of effecting the rapid detoxication of excessive amounts of tyramine absorbed from the intestine. In this connection it is worthy of note that rabbit liver has been found to contain the most rapid and efficient tyramine-oxidising system, and this may be correlated with the fact that rabbits have a very large caecum, which probably harbours an unusually large number of bacteria, and these may produce more tyramine than those in the intestine of a carnivore.

Secondly, it is possible, though unlikely, that tyramine or some nearly related compound is formed during the normal catabolism of tyrosine and then may be oxidised by this enzyme. A decarboxylating mechanism for tyrosine is however not yet known, and such a process would involve the postulation of an entirely new mode of catabolism for the amino-acids.

Thirdly, it is possible that this oxidative deamination is connected with the synthesis of some substance which is necessary for the animal body. There are certain facts, stated in the experimental section, which lead to the

idea that the end-product of the reaction is not the simple acid, *p*-hydroxy-phenylacetic, isolated from perfusion fluids by Ewins and Laidlaw. Particularly, they failed to find the acid after incubation of liver extracts and tyramine, and the incomplete deamination which occurs suggests that the final product cannot be free from nitrogen. Possibly, two molecules of tyramine condense, forming a dipeptide linkage, or, two nuclei may join together by means of an ether linkage, and the nitrogen of one molecule may remain unattacked as an amine side chain.

There are several very interesting properties exhibited by the tyramine oxidase system. It resembles the xanthine oxidase in being unaffected by the addition of cyanide, and thus forms another exception to Warburg's statement that no direct oxidation can proceed in which atmospheric oxygen is not "activated" by iron. The production of hydrogen peroxide during the reaction seems to show that molecular oxygen acts as a direct acceptor of hydrogen in the system. Whether or not any possible correlation exists between these two facts is still uncertain. The possible significance of the occurrence of hydrogen peroxide during the process of an oxidation has been fully discussed by Thurlow.

On the other hand, it differs from the above system in that, whereas the latter readily reduces methylene blue, the tyramine oxidase system apparently cannot utilise this dye as a hydrogen acceptor in the place of atmospheric oxygen. In this property it resembles the "aerobic oxidases," tyrosinase and the phenolases. Whether this difference between the two classes of enzymes is due to a fundamental difference in the mechanism of their action, or is due to our lack of knowledge of the oxidation-reduction potentials concerned, is at present undecided. The tyramine oxidase system, however, occupies a unique position and cannot as yet be classed with any of the known types of enzymes. The significance of the system cannot be properly discussed until the end-products of the reaction have been identified.

#### SUMMARY.

1. Using the Barcroft technique, an oxidation of tyramine (*p*-hydroxy-phenylethylamine) has been shown to take place by means of an enzyme contained in extracts of liver. The oxygen uptake corresponds to the absorption of one atom of oxygen per molecule of tyramine. Methylene blue, however, is not reduced.

2. It has been shown that deamination occurs simultaneously with the oxidation, but only half the nitrogen present in the tyramine molecule can be accounted for as ammonia.

3. The enzyme can be obtained in a cell-free solution.

4. The oxidation is not affected by the addition of *M*/500 KCN.

5. The production of hydrogen peroxide during the reaction has been demonstrated.

6. The influence of hydrogen ion concentration on the system has been studied, and the optimum reaction has been shown to be at  $p_H$  10.0. The enzyme is inactive, but not destroyed, at  $p_H$  4.4, but destruction occurs at  $p_H$  11.5.

7. Phenylethylamine and possibly also *p*-aminophenol are oxidised in the presence of liver extracts, but tyrosine and many other substances are unattacked. The system produces no pigment and in many ways is shown to be distinct from tyrosinase.

8. The possible significance of the enzyme system is discussed.

My thanks are due to Sir F. G. Hopkins for his continual help and encouragement, and to Dr M. Dixon for valuable suggestions and criticism. I am indebted to Dr P. P. Laidlaw for a supply of *p*-hydroxyphenylacetic acid.

## REFERENCES.

- Abderhalden and Gebelcin (1926). *Z. physiol. Chem.* **152**, 125.  
Battelli and Stern (1912). *Biochem. Z.* **46**, 317.  
Bernheim and Dixon (1928). *Biochem. J.* **22**, 125.  
Bertrand (1907). *Compt. Rend. Acad. Sci.* **145**, 1352.  
Dale and Dixon (1909). *J. Physiol.* **39**, 25.  
Dixon (1925). *Biochem. J.* **19**, 672.  
Ewins and Laidlaw (1910). *J. Physiol.* **41**, 78.  
Hiller and van Slyke (1922). *J. Biol. Chem.* **53**, 253.  
Johnson and Daschavsky (1925). *J. Biol. Chem.* **62**, 725.  
Lehmann (1909). *Arch. Hyg.* **67**, 99.  
Szent-Györgyi (1924). *Biochem. Z.* **150**, 195.  
Thurlow (1925). *Biochem. J.* **19**, 175.  
Warburg (1921). *Biochem. Z.* **119**, 134.  
—— (1923, 1). *Biochem. Z.* **136**, 266.  
—— (1923, 2). *Biochem. Z.* **142**, 518.

## CXXII. THE EXAMINATION OF IRRADIATED ZYMOSTEROL FOR THE PRESENCE OF VITAMIN D.

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It has recently been shown that ergosterol is associated in yeast with a highly unsaturated dextrorotatory sterol to which the name of zymosterol has been given [Smedley-MacLean, 1928]. This sterol appears closely to resemble ergosterol in its chemical properties; both contain three ethylenic linkages, both are readily susceptible to the oxidising action of air acting in the presence of light; they differ however markedly in their physical properties. Unlike ergosterol, a solution of zymosterol in alcohol produces no selective absorption bands in the ultra-violet region and, whereas ergosterol is strongly laevorotatory in ether or chloroform solution, zymosterol is dextrorotatory. Although the absence of selective absorption in the ultra-violet region rendered it unlikely that the new sterol could change on irradiation with ultra-violet light into a biologically active substance, it was desirable to establish this result by direct experiment.

The zymosterol was prepared from the mixture of sterols obtained on saponification of yeast fat and was separated from the ergosterol present in the mixture by fractional crystallisation. At present it has not been found possible to obtain a specimen of zymosterol entirely free from ergosterol; since both sterols are precipitated by digitonin, the amount of the impurity present was estimated by examination of the absorption spectrum in the ultra-violet region. The effect of a solution of one part of zymosterol in 5000 parts of alcohol was compared with that of solutions of different concentrations of ergosterol, and the amount of ergosterol present as impurity was taken as that present in the solution of pure ergosterol which produced the same absorption spectrum as the zymosterol solution. The spectroscopic examination showed that the specimen of zymosterol used in the first feeding experiment contained as much as 12 % of ergosterol. Subsequently a specimen was obtained in which the impurity was reduced to less than 5 %. The irradiation was carried out for 1 hour in 1 % ethereal solution at a distance of 32 cm. from a mercury vapour quartz lamp (Hewittic Company). The irradiated sterol was added to

an amount of hardened cotton-seed oil calculated so that a drop of the oil from a standardised pipette contained the minimum dose of irradiated sterol to be given.

#### BIOLOGICAL EXPERIMENTS.

For the biological tests, young rats were used and before a conclusive result was obtained four different diets had been employed. These were (1) a diet (*F* diet [see Hume, Smith and Smedley-MacLean, 1928]) deficient only in the fat-soluble vitamins A and D; (2) the same diet to which 20 % wheat germ had been added [see Leigh-Clare and Soames, 1928] to provide vitamin A; (3) diet 3143 of McCollum [McCollum *et al.*, 1921]; and (4) diet 84 of Sherman and Pappenheimer [1921]; the last two are both incomplete diets with a very low proportion of phosphorus. The percentage ash in the dried extracted bone and the degree of alkalinity of the faeces were examined in the rats fed on all the four diets. The method used for the examination of the faeces was a modification of that described by Jephcott and Bacharach [1926]; 0.2 g. faeces was rubbed up with 10 cc. distilled water and the hydrogen ion concentration determined by means of the capillator, bromothymol blue and phenol red being used as indicators. The results obtained in one series of readings were compared with those obtained by use of the hydrogen ion concentration cell and were found to be in close agreement. On the McCollum diet the degree of macroscopic rickets in the rib junctions was noted and on the Sherman-Pappenheimer diet an X-ray photograph of the right leg of each rat was taken as described by Rosenheim and Webster [1926].

#### *Experiments using F diet.*

Young rats of about 40 g. weight were placed upon the diet and after a period of from 4 to 8 weeks the weight curve became flat, showing that the reserve of vitamin D was exhausted; at this point the doses of irradiated sterol to be tested were administered daily for about 5 weeks. The rats retained a sufficient reserve of vitamin A to carry them through the experiment. Doses of 1/5,000, 1/10,000 and 1/20,000 mg. of irradiated ergosterol and zymosterol were used, and at the end of the experiment the percentage ash in the dried extracted leg bones was compared with that of untreated controls. As the zymosterol was never obtained free from ergosterol and might be owing all its activity to the impurity, the doses of irradiated sterols have been set out in Table I so as to show the dose of ergosterol given as impurity in each dose of zymosterol and these have been included in the series of ergosterol doses, each such dose appearing in the table under the dose of zymosterol of which it really formed a part. The doses of 1/5,000, 1/10,000 and 1/20,000 mg. of the first specimen of zymosterol used contained also doses of 1/40,000, 1/80,000 and 1/160,000 mg. of irradiated ergosterol respectively.

Reference to the table shows that the doses of 1/5,000 and 1/10,000 mg. of irradiated ergosterol and the same doses of zymosterol (containing 1/40,000



Table I. Showing percentage of ash in dried extracted leg bones of rat.

[illegible]

\* These ash determinations were calculated on the dried bone before it had been extracted and gave therefore too low values. The terms "severe," "slight," etc. refer to the degree of rickets estimated by macroscopic inspection of rib junctions.

and 1/80,000 mg. ergosterol) all exercised a definite influence on calcification. Unfortunately only two animals were available for comparing the doses of 1/20,000 mg. ergosterol and 1/20,000 mg. zymosterol (containing 1/160,000 mg. ergosterol), but as far as such evidence goes it shows that the dose of 1/20,000 mg. ergosterol was effective but not the similar dose of zymosterol.

This simple comparison, however, suggested that the zymosterol was at least less effective than the ergosterol, and supposing that it was not effective at all indicated that the minimum dose of irradiated ergosterol which would exercise a definite effect upon calcification in rats receiving the *F* diet lay somewhere between 1/80,000 and 1/160,000 mg. Examination of the alkalinity of the faeces showed that, with rats fed on the *F* diet, there is not the same sharp differentiation between the controls and the rats receiving the doses of irradiated sterols as there is in rats fed on the less complete McCollum or Pappenheimer diet, although even on the *F* diet the faeces of the rats receiving irradiated sterol were on the whole more acid than those of the controls.

*Experiments with F diet, containing 20 % wheat germ.*

The experiment was begun as in the preceding instance on the *F* diet only, but the rats proved to have so large a reserve of vitamin D that by the time this was exhausted the reserve of vitamin A had also almost come to an end. In order to supply vitamin A, the diet containing 20 % wheat germ, which is a modification of the *F* diet, was therefore substituted after the rats had been 10 weeks on the *F* diet. Doses of 1/100,000 and 1/200,000 mg. irradiated ergosterol and the same doses of irradiated zymosterol (containing  $\frac{1}{2 \times 10^6}$  and  $\frac{1}{4 \times 10^6}$  mg. ergosterol) were then given daily for 42 days. At the end of that time the dried extracted bones were ashed and the percentage ash compared with that of controls. Reference to Table I shows that on this diet, complete except for the lack of vitamin D, the addition of even very small doses of irradiated sterol produced a marked effect. The effect of the dose of 1/100,000 mg. irradiated ergosterol is somewhat better and the dose of 1/200,000 mg. very definitely superior to that of the corresponding dose of irradiated zymosterol, but, though inferior, it is still not possible to say that the irradiated zymosterol has no activity of its own. The smallest dose of impurity is here one four-millionth milligram of irradiated ergosterol and, supposing the zymosterol to have no activity of its own, an effect on calcification would have to be ascribed to this extremely small dose, when added to this particular diet.

There was no sharp differentiation between the degree of alkalinity of the faeces of the controls and those of the rats receiving doses of the irradiated sterols. In most cases the  $p_H$  did not exceed 6.8.

*Experiments with McCollum's diet 3143.*

Since a response was obtained with such small doses of sterol on the wheat germ diet, making it almost impossible to find sharply the minimum dose, it

was thought that perhaps better differentiation would be shown on a diet of a more severe type. Diet 3143 of McCollum was therefore used after the rats had been for a fortnight on the *F* diet without wheat germ, it having been first intended to use that diet again. After 14 days on the *F* diet, the rats received the McCollum diet together with the dose to be tested over a period of 4-5 weeks.

Daily doses of  $1/100,000$  and  $1/400,000$  mg. of irradiated ergosterol were given and the same doses of irradiated zymosterol containing respectively  $\frac{1}{2 \times 10^6}$  and  $\frac{1}{8 \times 10^6}$  mg. ergosterol.

Macroscopic observations of the degree of rickets in the rib junctions were made and the percentage of ash in the dried extracted bone was also determined.

The results obtained in this experiment showed clearly that the only dose which produced any evidence of a definite protective action was the  $1/100,000$  mg. of ergosterol. The effect of the  $1/400,000$  mg. ergosterol was very slight but it seemed definitely to be somewhat better than that of either of the doses of zymosterol, the value of which appeared closely to resemble that of the controls.

With rats fed on the McCollum diet the inference could be drawn that the minimum dose of irradiated ergosterol which produces a significant effect is somewhere about  $1/100,000$  mg. and lies between this and  $1/400,000$  mg.

The faeces of all rats on this diet developed marked alkalinity ( $p_{H}$  7.4 to 8.4); those of the rats receiving the  $1/100,000$  mg. of irradiated ergosterol were all less alkaline than those of the remaining animals, amongst which there appeared to be no significant differences.

#### *Experiments with the Sherman-Pappenheimer diet.*

It still remained uncertain whether zymosterol after irradiation had a certain value, though less than one-fourth that of an equal dose of irradiated ergosterol, or whether such activity as it acquired was due to the ergosterol present as impurity. An experiment was therefore carried out using the method of Rosenheim and Webster [1926] in which the rats are fed on the Sherman-Pappenheimer diet and an X-ray examination is made of the leg bones. These workers have examined a large number of rats with varying doses of ergosterol and have found that the usual minimum dose necessary to give a normal X-ray picture lies in the region of  $1/10,000$  mg. irradiated ergosterol [Rosenheim and Webster, 1927].

Young rats weighing 38 to 43 g. were used and arranged in four comparable groups, each containing six animals. One group was retained as controls, each animal of a second group received  $1/5,000$  mg. irradiated zymosterol, the animals of the third group were given  $1/20,000$  mg. irradiated ergosterol, while the rats in the last group were given  $1/100,000$  mg. irradiated ergosterol. Spectroscopic examination of the specimen of zymosterol used in this experiment had shown that it contained not more than 5 % ergosterol and therefore

a dose of 1/100,000 mg. of ergosterol corresponded to the maximum amount of this substance which could be present as impurity in the dose of zymosterol actually given. The animals received the diet and the daily dose for 25 days from the start of the experiment; they were then killed, the right leg dissected off and an X-ray picture taken. The result of this examination was conclusive (see Plate II); the controls gave a picture of severe rickets, the rats receiving 1/20,000 mg. ergosterol were almost normal while the bones of those receiving 1/5,000 mg. zymosterol (containing 5 % ergosterol as impurity) and 1/100,000 mg. ergosterol were indistinguishable. Any effect produced by the specimen of irradiated zymosterol used could therefore be ascribed to the effect of the ergosterol contained in it as impurity, and there was no evidence that the irradiated zymosterol was biologically active.

An estimation of the ash in the dried extracted bones gave the rather curious result that the rats receiving all three doses of the two sterols showed a definitely better calcification than the controls, but showed no differentiation among themselves. It seems possible therefore that on a diet such as the Sherman-Pappenheimer, very defective in phosphorus, a very small dose of vitamin D is sufficient to increase the percentage of ash in the bone but a much larger dose of this vitamin is not able further to increase this percentage in spite of the fact that the bone may give an almost normal appearance of calcification when the X-ray examination is made.

#### DISCUSSION.

Besides establishing the fact that zymosterol is not capable of being activated by ultra-violet irradiation as ergosterol can be activated, the present series of experiments provides a basis of comparison for the minimum doses of irradiated ergosterol necessary to produce an effect when different diets are used and shows the results obtained by different methods of testing for vitamin D. When the more complete diets are given, the estimations of ash do not apparently differentiate sufficiently sharply between the effects of the various doses of the two sterols, or rather there is not sufficient difference shown over a large range of dosage. With diets deficient only in the fat-soluble vitamins, a dose of 2-8 millionths of a milligram of irradiated ergosterol appears to produce some effect on the percentage of ash in the bone, but increase of this dose produces very little more effect until a much larger dose is reached. At the same time in this series of experiments the inferiority of the irradiated zymosterol was definitely indicated.

The experiment using the McCollum diet in which only very small doses of the sterols were used gave a quite definite result; the X-ray test carried out on the rats fed on the Sherman-Pappenheimer diet was certainly the most conclusive. In judging between the methods it must, however, be remembered that the last-named method was used in the final experiment when the results of the previous experiments were available, and that this method having been extensively made use of by Rosenheim and Webster their wide experience as

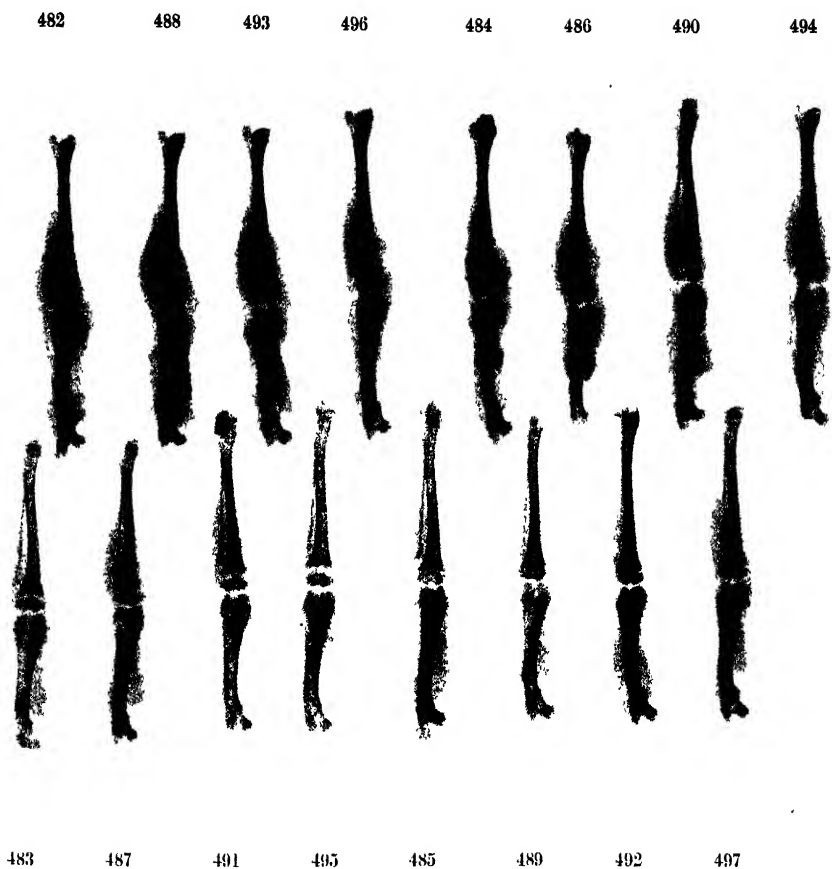
to dosage was available when the experiment was planned. In testing for the presence of vitamin D the use of the McCollum or Sherman-Pappenheimer diet appears to give more sharply differentiated results and to have the advantage of being more quickly carried out. With these severe diets, the minute doses which appear to have a definite effect in increasing the percentage of ash in the bone produce no result. With the McCollum diet a marked effect was produced by a dose of 1/100,000 mg. of the irradiated ergosterol though this dose was insufficient to ensure complete protection. Using the Sherman-Pappenheimer diet, our experience agreed with that of Rosenheim and Webster that a dose of rather more than 1/20,000 mg. was necessary to give a normal picture.

The results of the examination of the faeces were in general agreement with the other methods of testing above described when the Sherman-Pappenheimer or McCollum diet was used, but were not sufficiently sharply differentiated when the more complete diet was given.

We wish to express our thanks to Dr Rosenheim and Mr Webster for their help in arranging for the X-ray examinations of the bones and to Dr N. S. Lucas for carrying out for us the spectroscopic examinations necessary to determine the percentage of the ergosterol present in our specimens of zymosterol. We desire also to acknowledge our indebtedness to the Medical Research Council and to the Department of Scientific and Industrial Research for grants which have enabled us to carry out this investigation.

#### REFERENCES.

- Hume, Smith and Smedley-MacLean (1928). *Biochem. J.* **22**, 27.  
 Jephcott and Bacharach (1926). *Biochem. J.* **20**, 1351.  
 Leigh-Clare and Soames (1928). *Lancet*, i, 150.  
 McCollum, Simmonds, Shipley and Park (1921). *J. Biol. Chem.* **47**, 507.  
 Rosenheim and Webster (1926). *Biochem. J.* **20**, 537.  
 ——— (1927). *Lancet*, ii, 621.  
 Sherman and Pappenheimer (1921). *Proc. Soc. Exp. Biol. Med.* **18**, 193.  
 Smedley-MacLean (1928). *Biochem. J.* **22**, 22.



Results of X-ray examination of leg bones of rats on Sherman-Pappenheimer diet.

497, 492, 489 and 485. Rats received 1,20,000 mg. irradiated ergosterol daily.

495, 491, 487 and 483. Rats received 1.5,000 mg. irradiated zymosterol.

482, 488, 493 and 496. Rats received 1/100,000 mg. irradiated ergosterol.

484, 486, 490 and 494. Controls.



# CXXIII. THE ABSORPTION SPECTRUM OF VITAMIN A.

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*(Received June 30th, 1928.)*

THE absorption spectrum of cod-liver oil possesses a quite special interest because of the fact that the oil contains three known important substances which are sensitive towards light, namely, vitamin A, ergosterol or provitamin D and vitamin D itself.

A number of workers have studied the absorption spectrum of the medicinal oil, and Schlutz and his colleagues [1925, 1926] and Heilbron, Kamm and Morton [1927, 1] agree that selective absorption occurs in the regions  $320\text{--}328\mu$  and  $270\text{--}290\mu$ . Woodrow [1928], using a photoelectric spectrophotometer, has been able to find not only a band at about  $320\mu$ , but has also recorded the three absorption bands of ergosterol in thin films of the oil. The latter observation is of special interest as demonstrating the extreme sensitiveness of the spectroscopic method under favourable conditions. Even the cholesterol fraction from cod-liver oil contains only about 1 part in 4000 of ergosterol, and the proportion of cholesterol in the cod-liver oil is only some 0.5 %. Moreover, Woodrow's work definitely shows that the absorption band near  $270\text{--}290\mu$  is sometimes, if not necessarily, associated with provitamin D.

Nearly two years ago we adopted the following working hypotheses:

(a) that provitamin D should show selective absorption in the near ultra-violet;

(b) that vitamin A should show selective absorption in the nearer ultra-violet, since it has been demonstrated by many workers, *e.g.* Peacock [1926], that vitamin A may be destroyed by sunlight or the light from an incandescent lamp, and especially by the radiation from a quartz mercury lamp.

The first hypothesis was confirmed by a spectrographic study of cholesterol from brain or from cod-liver oil. Three absorption bands were shown to be a criterion of provitamin D and full use of these observations has been made in the later work by various authors on the photochemical conversion of ergosterol into vitamin D. In order to test the second hypothesis, it has been necessary to collect samples of materials varying widely in respect of the proportion of vitamin A present.

Since cod-liver oil remains the best-known source of the growth-promoting factor, it seems reasonable to test the assumption that the selective absorption at  $320\text{--}328\mu$  is connected with the vitamin. In most medicinal oils it is only



possible to record a marked inflexion at this point. If, however, samples of unrefined oil (obtained from fresh livers) are examined, it is possible to observe a real, well-defined absorption band. We have been fortunate in obtaining from Professor T. P. Hilditch samples of untreated oil from livers 1 day after their removal from the fish, and on examination we have found that very distinct selective absorption is shown in the near ultra-violet, the band exhibiting a clear maximum at  $328.5\mu\mu$ . It is interesting and significant that the oil gives an unusually good test with the antimony trichloride reagent which is generally regarded as being almost specific for vitamin A.

By the kindness of Professor J. C. Drummond we have been able to examine three other specimens of cod-liver oil differing widely in respect of vitamin A potency. An examination of these has shown that the intensity of the  $328\mu\mu$  absorption band varies directly with the depth of the blue colour obtained with the antimony trichloride test.

Up to this point the parallelism between the colour test and the absorption band seems unmistakable, but it might be argued that the agreement is fortuitous, arising from the fact that all the observations were carried out on cod-liver oil. Difficult as such an objection might be to sustain, the possibility of a coincidence could not be overlooked. The examination of a large number of liver oils from various marine animals appears, however, definitely to have eliminated any such objection. In some cases oils were used which showed by the colour test no signs of the presence of vitamin A, and in such it proved impossible to record a definite inflexion in the absorption curve in the region of  $320\text{--}328\mu\mu$ . Other oils were rich in the chromogen reacting with antimony trichloride to give the blue colour, and with these in every case the  $328\mu\mu$  absorption band was evident. Further, as is clearly indicated in the accompanying table, a strict correspondence is manifest between the intensity of the blue colour and that of the absorption band.

At this stage two apparent difficulties may be explained. In a dogfish-liver oil known to be rich in vitamin A, both by the colour test and by feeding experiments, the definition of the  $328\mu\mu$  absorption band was apparently poor. It will be noted that in the region of  $280\mu\mu$  (see Fig. 1) the oil shows relatively high selective absorption, so that actually two absorption bands are superimposed in the ultra-violet, and the  $328\mu\mu$  band is partially masked. When, however, the oil is "blown" with oxygen for 1 hour at  $100^\circ$  a marked change takes place. Instead of the characteristic blue colour with antimony trichloride, a red colour is at once obtained. The absorption spectrum shows a marked increase in transmission over the whole region  $270\text{--}350\mu\mu$ . If now the curve for the "blown" oil is subtracted from that for the crude oil, the result is a well-defined absorption band with a maximum near  $320\mu\mu$ . The investigation of this oil demonstrates clearly that the absorption band can only be used as a measure of the vitamin A content if due care is observed in allowing for the part played by any substances absorbing strongly in a neighbouring region of the spectrum. In this connection it should be pointed out

that column (5) in Table I (p. 993) may not always represent strictly quantitative data, and that in this communication we are seeking merely to establish the generalisation that the absorption band is a true characteristic of the vitamin.

The other difficulty is that we have observed [1927, 2] a band at  $318\text{--}319\mu\mu$  in certain samples of ground-nut (arachis) oils. Vegetable oils are often considered to be devoid of vitamin A, but Bacharach [1928] has proved that soya bean oil may be a good source. Whilst it is quite possible that arachis oils may contain vitamin A, the quantity present cannot exceed 5 % of that present in cod-liver oil. The difference between  $319$  and  $328\mu\mu$  may be due to solvent shift, since alcoholic solutions are used in all cases other than arachis oil, which is used without solvent.

Various processes have been proposed for the extraction of vitamins from cod-liver oil. The well-known proprietary article "ostelin" is definitely rich in vitamin D, but is not claimed to be rich in vitamin A. Judged by the presence of an inflexion in the absorption curves of certain samples it is not always entirely free from vitamin A and this is in agreement with the method of manufacture which is such as to make it quite likely that traces of the latter will occasionally be present. Another commercial preparation, namely "essogen," gives a marked reaction with antimony trichloride, and also shows very clearly the absorption band at  $328\mu\mu$ . In addition to the cod-liver oils already mentioned, Professor J. C. Drummond has also sent us samples of vitamin A concentrates prepared in his laboratories. The materials were "unsaponifiable" extracts from cod-liver oil and sheep-liver fat, and were free from sterols precipitable by digitonin. The intensity of the  $328\mu\mu$  band indicated that the cod-liver oil extract was 200–300 times, and the sheep-liver fat extract 800–1000 times, as potent as a typical cod-liver oil. Professor Drummond's independent estimates were 500 and 775, the figures being based on the colour test.

The correlation between vitamin A and an absorption band at  $328\mu\mu$  would therefore seem to be proved. The connection can, however, be subjected to even stricter tests. Vitamin A is known to be easily destroyed by oxidation, and if our contention is justified this process should simultaneously destroy also the chromogen responsible for the antimony trichloride blue and effect the disappearance of the  $328\mu\mu$  absorption band. Professor Hilditch has supplied us with samples of cod-liver oil "blown" under different conditions. Aeration at  $40^\circ$  for 4 hours resulted in no perceptible decrease either in the intensity of the blue colour, or in the persistence of the absorption band. On the other hand, aeration for 4 hours at  $80^\circ$  resulted in a very noticeable change, reflected to the same extent by the colour test and by the spectroscopic criterion. We have also found that after treatment of "essogen" or shark-liver oil with oxygen at  $100^\circ$  for 2 hours, the absorption band had entirely disappeared and the blue colour was so feeble as to be negligible.

The instability of vitamin A towards light makes it necessary to demonstrate not only that the vitamin can be destroyed by irradiating an oil, but

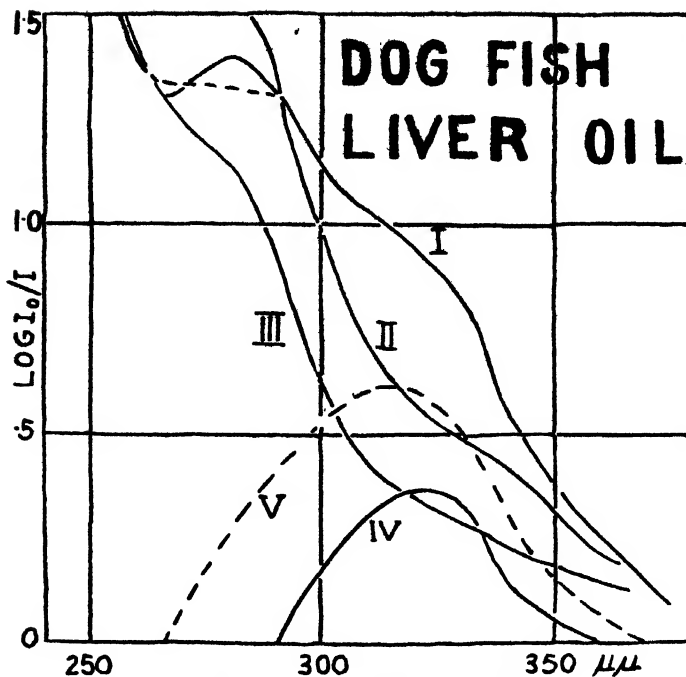


Fig. 1. Absorption spectra of dogfish-liver oils.

- I. Untreated oil 1% solution 3 mm. cell.
- II. Same oil "blown" with oxygen for 1 hour at 100°, 1% solution 3 mm.
- III. Same oil exposed to sunlight in a quartz vessel.
- IV. 320  $\mu\mu$  band obtained by subtracting II from I.
- V. Absorption of materials destroyed by irradiation, probably vitamin A and ergosterol: curve obtained by subtracting III from I.

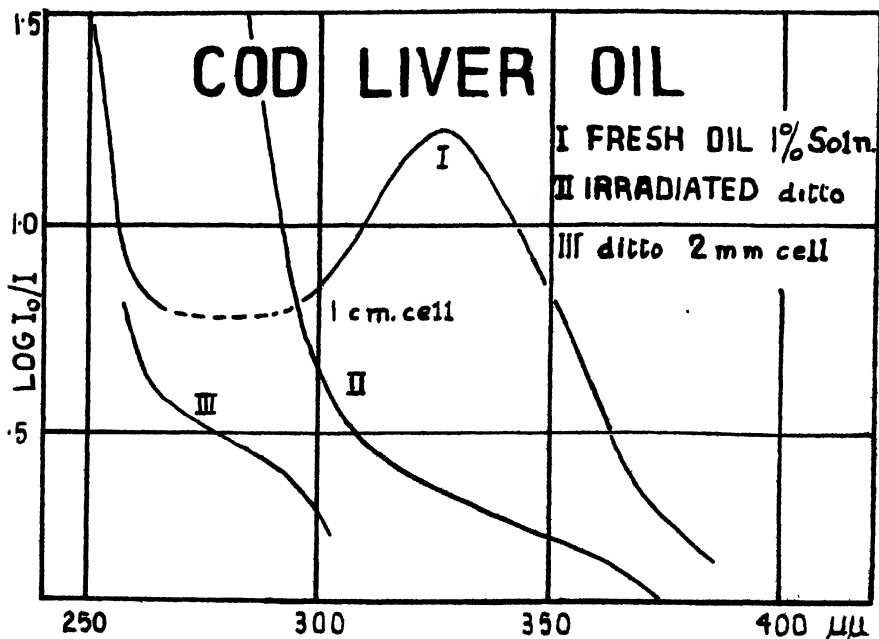


Fig. 2.

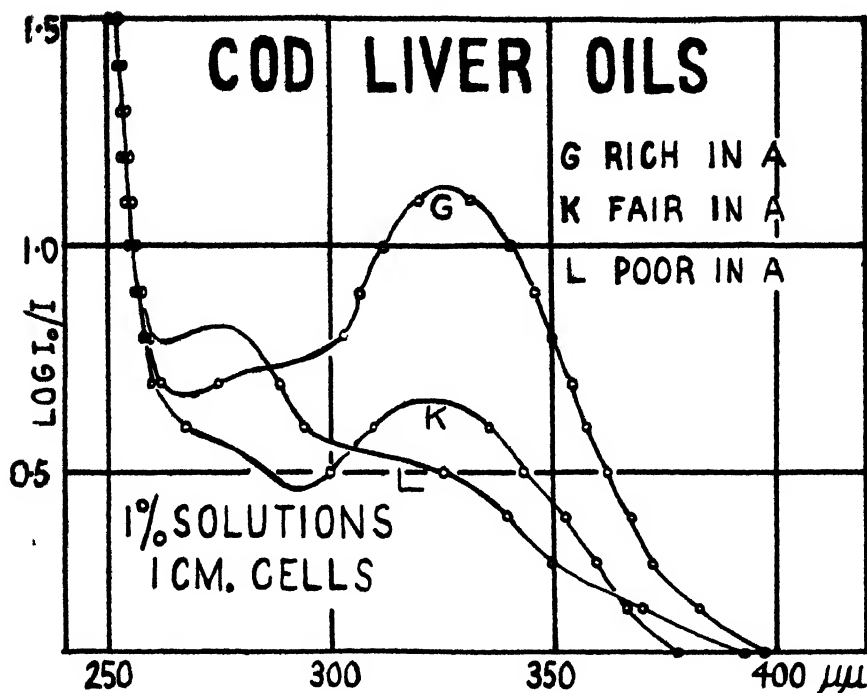


Fig. 3.

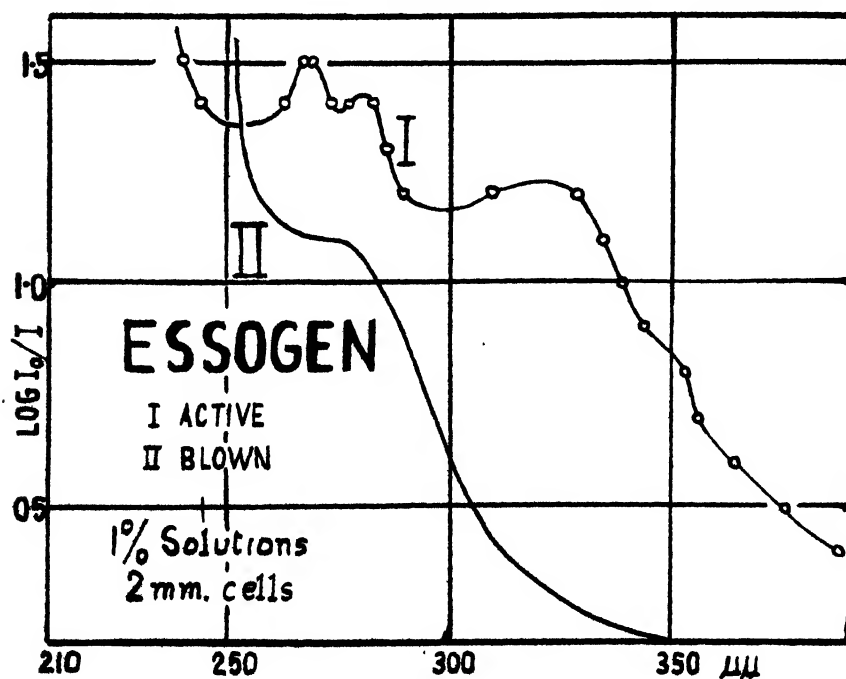


Fig. 4.

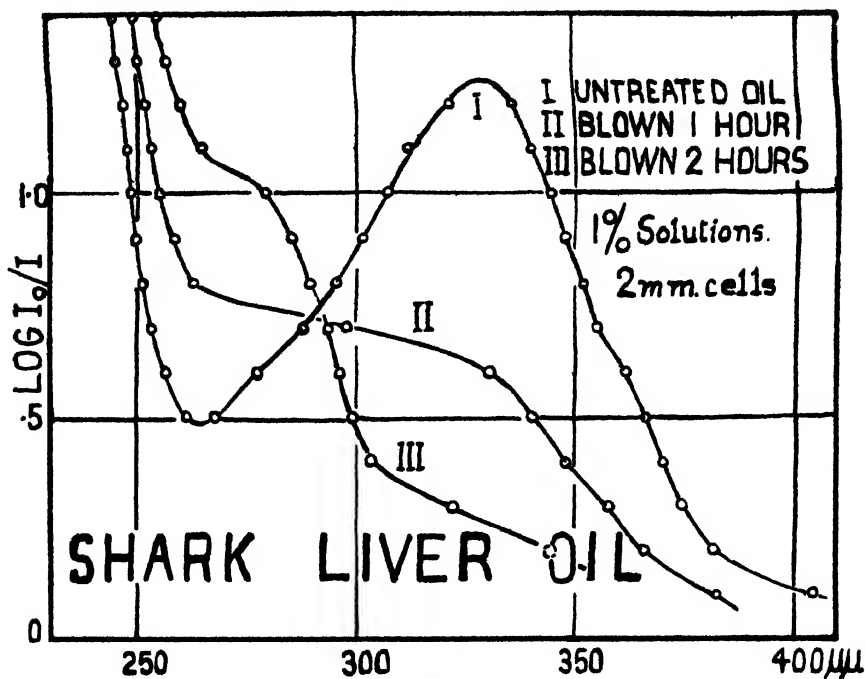


Fig. 5.

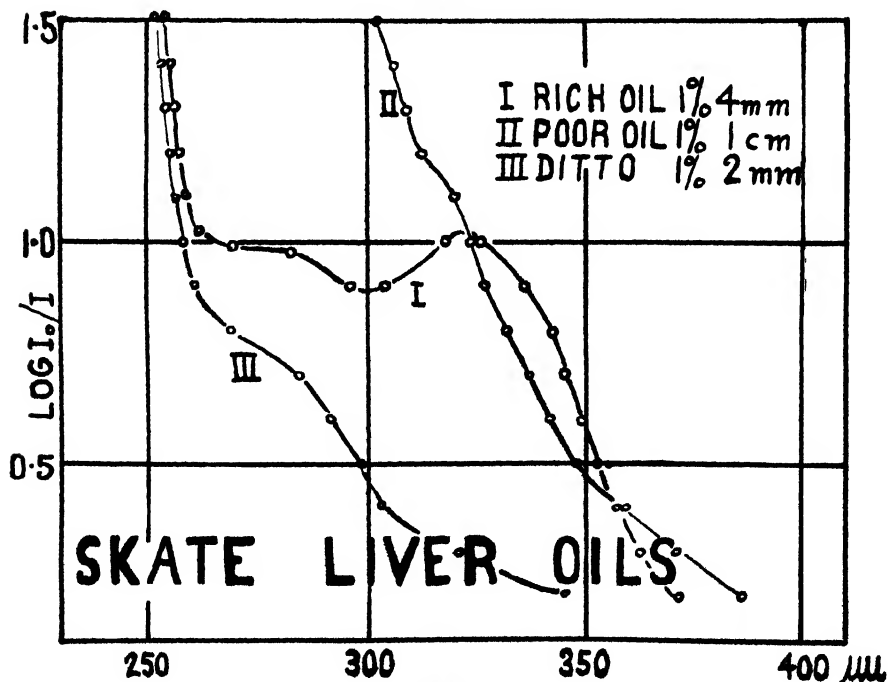


Fig. 6.

Table I.

Material	Band or inflexion near 230 $\mu\mu$	Band or inflexion near 270-290 $\mu\mu$	Band or inflexion 320-328 $\mu\mu$	Units of absorption*	Units of "blue"†
Marine animal oils of known origin:					
(1) <i>Hezanchus</i>	—	Faint inflexion	Absent	0	0
(2) <i>Spirax niger</i>	—	Definite	Absent	0	0
(3) <i>Scyllium canicula</i>	—	Faint	Absent	0	0
(4) <i>Scymnodon ringieris</i>	—	Faint	Doubtful	0	0
(5) <i>Raja clavata</i>	Inflexion	Definite	Absent	0	0
(6) <i>Raja batia</i>	Inflexion	Definite	Absent	0	1.0 circa
(7) <i>Scymnorhinus ichia</i>	Inflexion at 240 $\mu\mu$	Marked at 275 $\mu\mu$			
Cod-liver oils from Prof. Hilditch:					
(1) Oil from fresh livers	—	Ergosterol bands	Good band	10.8	9.9
(2) Chilled and filtered oil	—	Masked if present	Good band	11.0	9.9 approx.
(3) Non-freezing oil	No inflexion	Masked if present	Good band	12.0	10.5
(4) Stearin-rich oil	—		Good band	10.0	10.5 approx.
Blown oils from fresh livers:					
4 hours at 43°	—	No change	No change	12	Not measured
4 hours at 80°	—	Definite inflexion	Less intense	6	Not measured
4 hours at 80°	—	Definite	Less intense	<2.5	Not measured
Irradiated oil:					
Fresh liver oil irradiated for 4 days	—	Definite	Absent	0	0
Cod-liver oils of high medicinal quality from Prof. Drummond:					
"G" Scotch oil from Aberdeen	—	Ergosterol inflexion 275	Good band	11.5	10.0
"K" Lofoten oil	—	Inflexion 275	Smaller band	6.8	6.6
"L 4" Lofoten oil	—	Ergosterol definite	Clear inflexion	3.2	3.3
Essogen (Lever Bros.)	—		Clear band	<112	Not measured
Essogen blown with oxygen for 1 hour	—	Clear inflexion	Absent	0	0
at 100°	Inflexion	Clear inflexion	Doubtful	?	?
Ostelin					

\* The arbitrary unit of absorption here used may be defined as follows: when  $\log I_0/I$  (intensities of incident and emergent light respectively) represents the extinction  $E$ , 1 unit of absorption for a 1 % solution and a 1 cm. cell corresponds with a persistence of  $E=0.04$  for the 328  $\mu\mu$  band.

† 1 unit is given by  $\log I_0/I=0.275$  at 608  $\mu\mu$  using 2 cm. cells, 0.3 cc. of 10 % cod-liver oil in chloroform plus 3 cc. of 30 % antimony trichloride in the same solvent. This unit stands in close relationship with Lovibond units.

— indicates that definite results were not obtained.

Table I (continued)

Material	Band or inflexion near 230 $\mu\mu$	Band or inflexion near 270-290 $\mu\mu$	Band or inflexion 320-328 $\mu\mu$	Units of absorption	Units of "blue"
<b>Shark-liver oils:</b>					
Sample (1)	Inflexion	Ergosterol (?) Inflexion	Inflexion	?	?
Sample (2)	—	Band at 280 $\mu\mu$	Very weak	<12.5	12
Sample (3)	—	Masked if present	Good band	<12.5	14.5
Sample (4)	Good inflexion	Masked if present	Good band	72	80
Sample (5)	Good inflexion	—	Good band	94	90
Irradiated shark oil (4) (silvered quartz test-tube)	—	Inflexion	Much weakened	Not measured	Not measured
Blown shark oil (5): (1) oxygen for 1 hour at 100° (2) oxygen for 2 hours at 100°	—	Inflexion	Weaker	Doubtful	13} doubtful
Porbeagle shark-liver oil	—	Marked inflexion at 275 $\mu\mu$ Small inflexion	Much weaker Very doubtful	Doubtful 0	11} values 0
<b>Skate-liver oils:</b>					
Sample (1)	—	Clear inflexion at 275 $\mu\mu$ Marked inflexion	Poor Good band	?	<3.5
Sample (2)	—	—	—	Order 25	19
<b>Dogfish-liver oils:</b>					
Sample (1)	—	Band at 280 $\mu\mu$	Inflexion (see Fig. 1)	38	28
Sample (1) (blown with oxygen 1 hour at 100°)	—	Band at 280 $\mu\mu$	No inflexion	0	?
Sample (1) (exposed to sunlight in a quartz vessel)	—	Faint inflexion	Weak if at all	?	8.5
Whale oil	—	Inflexion	Faint	?	?
Wool fat	—	Inflexion	Faint band	?	Positive
Spent cod-liver oil from the Zucker process	—	Inflexion	Small band	Order 2	Not measured
"Un-sap." from cod-liver oil after removal of all sterols precipitable by digitonin (from Prof. Drummond)	No inflexion	Masked if present	Good band	600-1000	1650
"Un-sap." from sheep liver similarly prepared (from Prof. Drummond)	No inflexion	Masked if present	Good band	2500-3000	2380

that the chromogen and the absorption band can also be made to disappear under the same circumstances. It will be seen from the figures and the accompanying table that irradiation of cod-liver oil has the expected effects.

In order to put the photochemical mechanism to a final test the following procedure was adopted. A 4-inch quartz test-tube of  $\frac{3}{8}$  inch external diameter was covered with a bright film of silver on the outside. The transmission spectrum of silvered quartz almost coincides with the  $328\mu\mu$  absorption band, so that if an oil rich in vitamin A is placed in the tube and subjected to the action of the light from a quartz mercury lamp, the only rays capable of reaching the oil are those within the absorption band. After 24 hours' irradiation, the blue colour with antimony trichloride was noticeably weaker, and after 48 hours' irradiation the very rich shark oil used gave a test little stronger than that given by an ordinary cod-liver oil. The intensity of the  $328\mu\mu$  absorption band had by this time decreased almost to zero, at the concentration used initially.

It will be noticed that in the table and figures many oils show an inflexion or band in the neighbourhood of  $275\text{--}285\mu\mu$ . In some of these oils, the vitamin had been destroyed during the experiments, and in others the colour test indicated the absence of the vitamin at the outset. We have recorded a similar band in fractions obtained from cod-liver oil concentrates, and it seems not at all unlikely that a decomposition product of vitamin A is characterised by an absorption band at  $275\text{--}285\mu\mu$ . In this connection it is interesting to recall the fact that bands in exactly the same region have been observed in un-saponifiable extracts from cotton-seed oil, and in some cases in vegetable oils themselves. Whether this is fortuitous or not is a matter which we have now under investigation.

We have also recorded in many oils an inflexion in the absorption curves at about  $230\mu\mu$ , but it has not so far proved possible to attach any significance to this band.

#### SUMMARY.

Hitherto there have been two criteria of vitamin A, the feeding test and the antimony (or arsenic) trichloride colour reaction. It is now claimed as a third property that vitamin A is characterised by an absorption band with a maximum at  $328\mu\mu$ .

It is probable that one of the decomposition products of vitamin A has an absorption band near  $275\text{--}285\mu\mu$ .

In conclusion we would like to express our thanks to Professors Drummond and Hilditch, to Mr A. L. Bacharach and Miss V. Hazley of the Glaxo Research Laboratory, and also to Messrs Creed, Gillam and Sexton for assistance in various directions. We are also indebted to the Food Investigation Board of the Department of Scientific and Industrial Research for supporting the investigation.\*



## REFERENCES.

- Bacharach (1928). *Quart. J. Pharm.* **1**, 49.  
Heilbron, Kamm and Morton (1927, 1). *Biochem. J.* **21**, 78.  
——— (1927, 2). *Biochem. J.* **21**, 1279.  
Peacock (1926). *Lancet*, ii, 328.  
Schlutz and Morse (1925). *Amer. J. Dis. Child.* **30**, 199.  
Schlutz and Zeigler (1926). *J. Biol. Chem.* **69**, 415.  
Woodrow (1928). *Phil. Mag.* 944.

## CXXIV. STUDIES ON COLOUR TESTS FOR STEROLS AND FOR VITAMIN A.

### II. A SPECTROSCOPIC STUDY OF THE COLORATIONS ATTRIBUTED TO VITAMIN A.

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*(Received July 2nd, 1928.)*

IN the first paper of this series an account was given of some sterol tests in which blue colours were obtained very similar in appearance to those given by arsenic trichloride or antimony trichloride with cod-liver oil, and attributed to vitamin A. On account of this close resemblance, it appeared desirable next to undertake a spectroscopic study of the "vitamin" colours with the object of discovering specific absorption bands by means of which the chromogen might be identified.

Examination of the literature brought to light two cases in which workers had previously examined this question. Rosenheim and Drummond [1925] mentioned that the absorption band of the blue colour given by the action of arsenic trichloride on cod-liver oil lies between 570 and 590  $\mu\mu$ . Euler, Myrbäck, Fink and Hellstrom [1927] have recently employed a spectrograph and Siegbahn registering microphotometer to record the absorption bands in the colours given on two samples of Norwegian cod-liver oil by both arsenic and antimony trichlorides. Their results with arsenic trichloride indicate the existence of two bands, one between 475 and 480  $\mu\mu$ , and a second at about 535  $\mu\mu$ . Both of these are well outside the range 570 to 590  $\mu\mu$  mentioned by Rosenheim and Drummond. In the case of antimony trichloride, again two bands were found, one between 475 and 482  $\mu\mu$ , and a second between 535 and 550  $\mu\mu$ .

Since these figures, which were the result of only two experiments with each reagent, showed a somewhat wide variation in themselves, and also did not agree with the earlier findings of Rosenheim and Drummond, it was considered desirable to carry out a more detailed study.

#### EXPERIMENTAL.

A Hilger E 3 quartz spectrograph was employed, with a sector photometer running at 80 to 120 revolutions a minute. This was standardised by photographing the spectrum from a mercury vapour lamp and observing on the plate the position of different lines in the mercury spectrum whose wave

lengths had been accurately determined by previous workers. By this means there was obtained a curve which could be used to correct readings taken at any part of the spectrum. The light source, kept at constant intensity, was an arc between iron and nickel electrodes, using a direct current of 4 amp. at 100 volt. The colours were examined in cells made from plane quartz plates separated by ground glass rings of about 20 mm. internal diameter and known thickness from 0.5 to 2 mm. Thus only two or three drops of liquid were required to fill the cells, and it was possible to work with quite small quantities of material. The cod-liver oils used as sources of the chromogen were from Norway, Newfoundland and Iceland, and each had been tested by animal experiments for its vitamin content, of which details are given below.

*Examination of "vitamin" colours given by arsenic and antimony trichlorides.*

One of the chief difficulties encountered in attempting the spectroscopic examination of the "vitamin" colours is their transient nature. Under ordinary conditions, the initial blue colour given by arsenic trichloride on cod-liver oil has practically disappeared within 2 or 3 minutes, and that given by antimony trichloride lasts little longer. On the other hand, characterisation of an absorption band by means of the spectrograph requires from 5 to 30 minutes, according to the depth of colour employed and the accuracy required.

In the case of arsenic trichloride, it has not yet been found possible to diminish the rate of fading of the "vitamin" colours, and the only method available has been to renew the colours every minute or so by mixing fresh quantities of oil and reagent, and photographing the mixture as rapidly as possible. Since the shape and size of the absorption bands vary with the nature and intensity of the colour, which in turn depend on the quantities taken and time after mixing, very careful control is needed to ensure that two sections of an absorption band given by two successive batches of oil and reagent will be properly superposable. As the result is not known until the whole of the exposures have been taken, and the plate developed, many failures may be experienced.

In practice the most satisfactory results were obtained by adding a given number of drops of oil from a standardised pipette to the measured volume of arsenic trichloride in a clean, dry test-tube, mixing rapidly by twirling, transferring to a clean, dry cell, and quickly placing the latter in position in the spectrograph, in which the sector was already revolving, and all adjustments made to enable exposure to be commenced without delay. After some experience, it was found possible to carry through all these steps, and commence taking photographs, within 30 to 40 seconds of mixing the oil and reagent. Euler and his co-workers do not state the time which elapsed between mixing and commencing exposure in the tests on cod-liver oil, but in the case of arsenic trichloride and arachis oil this interval is given as 90 seconds. Their results with cod-liver oil, therefore, apparently represent, not the initial colour

produced, but the blend of blue and red which has been shown by Wokes and Willimott [1927] to be present 2 or 3 minutes after mixing. In the present investigation, it was found possible by rapid working on constantly renewed mixtures to obtain the first eight photographs (6 to 60 seconds exposure, giving  $\log I_0/I$  values from 0.1 to 0.8) representing the "vitamin" colours as they appeared within not much more than a minute of mixing.

This method was then applied to a series of oils whose potency in vitamin A had been established by feeding experiments (see data in Table I). These comprised a Newfoundland oil, an Iceland oil both before and after refining, and a concentrate prepared by suspending in an inert oil the unsaponifiable

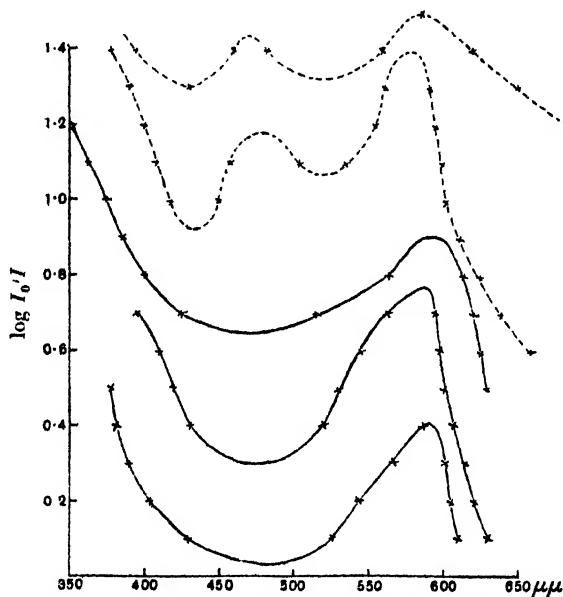


Fig. 1. Absorption curves of "vitamin" colours given by arsenic trichloride on cod-liver oils and concentrates.

- × - - × - - × Curves given by concentrates (suspensions of the unsaponifiable fraction of cod-liver oil in arachis oil).
- × — × — × Curves given by cod-liver oil.

matter from cod-liver oil. The curves are shown in Fig. 1, correction being made for the error due to the spectrograph, which was determined as previously described.

It will be seen that all the five oils gave a well-defined absorption band between 585 and 595  $\mu\mu$ , and that the two concentrates also showed a second band, due to a colour visually nearer the red, between 470 and 480  $\mu\mu$ . In Table I are given the approximate wave lengths for the head of each band, also the average figures for the two bands, which are 587 and 475  $\mu\mu$  respectively. From these results it appears that the blue colour produced by arsenic trichloride on cod-liver oil, and supposed to be due to vitamin A, can be characterised as showing an "initial" absorption band at about 587  $\mu\mu$ ,

and a "second" band, developing on standing, at about  $475\mu\mu$ . The concentrates, being several times more potent than the oils, gave specific absorption higher on the scale of  $\log I_0/I$  values, representing results obtained with longer exposures, when the "second" band also had time to develop.

Table I. *Results obtained with arsenic trichloride on cod-liver oils and concentrates.*

Source of oil	Approximate amount of oil taken* (cc.)	Feeding test for vitamin A	Wave lengths of heads of absorption bands ( $\mu\mu$ )
Newfoundland	0.12	+++†	— 594
Iceland, unrefined	0.06	++++‡	— 590
Iceland, refined§	0.12	++++	— 588
Concentrate, 1st	—	+++++	480¶ 580
Concentrate, 2nd	—	+++++	470¶ 585
Mean wave lengths			475 587

\* On account of the necessity for rapid working and frequent renewal of the reaction mixture, these quantities are only approximate.

† Normal growth, with complete freedom from xerophthalmia, in group of six rats, obtained with daily dose of about 10 mg.

‡ Normal growth, with complete freedom from xerophthalmia, in group of six rats, obtained with daily dose of about 5 mg. The feeding tests were carried out in co-operation with Dr S. G. Willmott. It has not yet been possible to determine accurately the minimum protective, or curative, doses, but these are certainly smaller than those given above.

§ The process of refining was by cooling, and did not involve exposure to heat and oxidation.

|| The concentrate was a suspension in an inert vegetable oil (arachis) of the unsaponifiable fraction of a potent cod-liver oil, but was not entirely free from cholesterol or ergosterol.

¶ These bands are quite distinct from those obtained by Euler at 440 or 445  $\mu\mu$  with arachis oil that had been standing in contact with arsenic trichloride.

On comparing these results with those of previous workers, it will be seen that the band observed between 570 and  $590\mu\mu$  by Rosenheim and Drummond [1925], but not found by Euler and his co-workers, has been confirmed in five separate cases, and its position more accurately defined as at about  $587\mu\mu$ . Further, the band found by Euler between 475 and  $480\mu\mu$  probably corresponds with the band found by the writer at about  $475\mu\mu$  in the case where time was allowed for it to develop. Euler's failure to distinguish the initial band at  $587\mu\mu$  was, no doubt, due to the longer interval he allowed between mixing oil and reagent and commencing to register the absorption bands in the changing colours produced.

On turning to the antimony trichloride reagent [Carr and Price, 1926] it was found possible to obtain much more sharply defined results. From a study of this reagent [Wokes and Barr, 1927] it was ascertained that the fading of the initial blue colour could be retarded by removal of moisture (amongst other factors); and, by applying a reagent made with well-dried chloroform to a stable oil, the characteristic blue colour has been made to persist in measurable amount (as measured by the tintometer) for nearly an hour. In this time there can be obtained from the one mixture of oil and reagent a sufficient number of exposures to characterise absorption bands with a considerable degree of accuracy.

Experiments similar to those with arsenic trichloride were then carried out on a number of oils of known vitamin A potency, as indicated in Table II, and from the plates thus obtained were plotted the curves shown in Fig. 2.

Table II. *Results obtained with antimony trichloride on cod-liver oils and concentrates.*

Source of oil	Approximate amount of oil taken* (cc.)	Feeding test for vitamin A	Wave lengths of heads of absorption bands ( $\mu$ )
Newfoundland	0.06	+++	— 615
Iceland, unrefined	0.06	++++	— 617
Iceland, unrefined	0.06	++++	— 617
Iceland, refined†	0.06	++++	— 612
Norwegian, refined	0.12	+++	— 610
Concentrate	—	+++++	530 612
Mean wave lengths			530 614

\* On account of the necessity for rapid working and frequent renewal of the reaction mixture, these quantities are only approximate.

† The process of refining was by cooling.

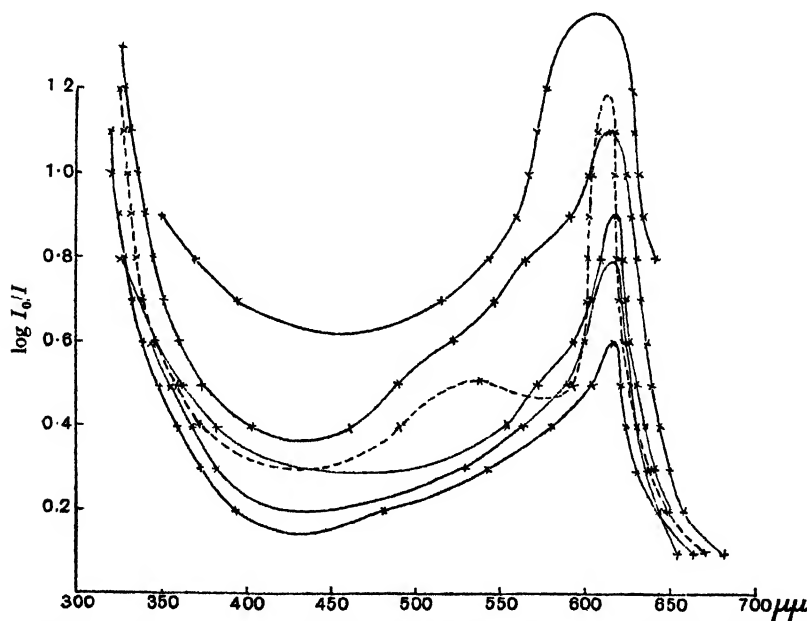


Fig. 2. Absorption curves of "vitamin" colours given by antimony trichloride on cod-liver oils and concentrates.

It will be seen that all the six samples gave a sharply defined band at about  $614\mu$ , and one in which more time was allowed for the reaction of the reagent gave a second less distinct band at  $525\text{--}535\mu$ . Confirmation of these results was kindly undertaken by Dr W. Brode, using the Hilger-Nutting spectrophotometer, which involves a somewhat different method [see Prideaux, 1926]. The head of the initial band was found by Dr Brode, working inde-

pendently, to be at about  $614\mu\mu$ , while on standing several minutes a second band was observed gradually to develop at about  $528\mu\mu$ . From these results it would appear that the blue colour given by antimony trichloride on cod-liver oil, and supposed to be due to vitamin A, can be characterised as showing an "initial" absorption band at about  $614\mu\mu$ , and a "second" band, developing on standing, at about  $528\mu\mu$ . In two cases some evidence was obtained of the presence of a further band or bands between 450 and  $500\mu\mu$ , but owing to the clouding of the mixtures on standing, the heads of the bands have not yet been accurately defined.

In the two curves published by Euler and his co-workers on a Norwegian oil with antimony trichloride, a sharp band is shown at 535 or  $550\mu\mu$ , and a very slight band at 475–482 $\mu\mu$ . The sharp band probably corresponds to the band found by the writer and by Dr Brode at about  $528\mu\mu$ , and the slight band to that between 450 and  $500\mu\mu$ . As with arsenic trichloride, the omission of Euler to record the initial band at about  $614\mu\mu$  is probably due to his allowing too long an interval between mixing and recording.

#### *Study of the time effect.*

Thus, in the case of both arsenic and antimony trichlorides, a spectroscopic study of the "vitamin" colours revealed the presence of at least two specific absorption bands. One, the "initial" band, was present at a very early stage in the reaction (less than a minute after mixing, under the given conditions); the other, "second" band, developed on standing.

The next question to be considered was whether there could be observed a decrease in the "initial" colour running parallel with the increase in the "second" colour, as the reaction proceeded. Some evidence of such a parallel change had already been obtained by Wokes and Willimott [1927] when examining by means of the tintometer different mixtures of oil and reagent. In a number of experiments with antimony trichloride it was found possible to measure in Lovibond units the relative amounts of blue and red colours at different times after mixing, and there was observed a gradual loss of blue colour occurring more or less simultaneously with a gain in red colour. There are, however, certain limitations in the Lovibond system which place difficulties in the way of such applications. Perhaps the most serious of these is the fact demonstrated by Gibson and Harris [1927] that the diminution in transmission of the glasses, on which depends their value as standards for colour measurement, extends over a considerable portion of the spectrum. The blue glasses, for instance, were found to give greatly reduced transmission between 570 and  $680\mu\mu$ , and the red glasses between 480 and  $590\mu\mu$ . Resort was therefore made to spectroscopic methods.

In the case of colours not altering appreciably in intensity over a period of say 15 to 30 minutes, the heights of their absorption bands, measured by means of the quartz spectrograph under given conditions, may be employed to ascertain the relative concentration of the chromogen; but since the study

of the "vitamin" colours by means of the tintometer had revealed a diminution in intensity of the initial blue colour of 10 to 20 % within 3 minutes after mixing, it became obvious that the ordinary spectrographic methods could not be employed.

This question of the decreasing intensity of the initial blue colour appears to have been overlooked by Euler and his colleagues. Euler makes the suggestion that the height of the absorption band produced can be used as a measure of the relative amount of vitamin present, and infers that antimony trichloride is more sensitive than arsenic trichloride because he found two drops of oil gave as high a band with antimony trichloride solution as three drops of the

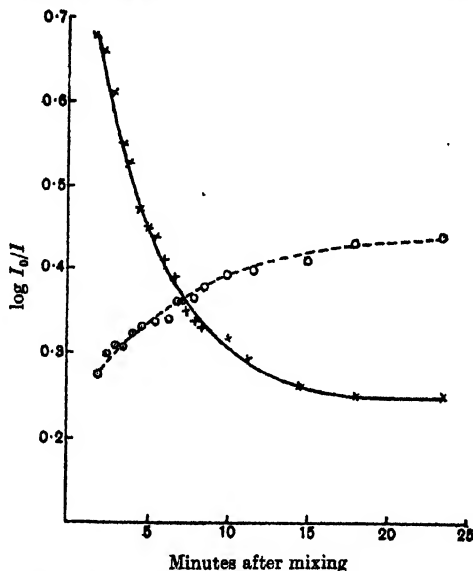


Fig. 3. Results obtained with the Hilger-Nutting spectrophotometer, showing changes taking place in the proportions of the two stages of the chromogen when left in contact with antimony trichloride.

x — x — x Amount of absorption at 614  $\mu\mu$ .  
o --- o --- o Amount of absorption at 530  $\mu\mu$ .

same oil with the same volume of arsenic trichloride. Since, however, one is dealing with colours which are changing continuously, no absorption bands of those colours can have any quantitative value unless the rate of change in colour intensity, and time elapsing between mixing and recording, are noted. In Euler's work this apparently has not been done.

From these considerations it seemed clear that, if a satisfactory study of these changing colours were to be made, a method would be required which allowed records of intensity of absorption to be made every half-minute or so. Such a method was found by adaptation of the Hilger-Nutting spectrophotometer. By means of this instrument it is possible to take as frequently as every 15 seconds a reading of intensity of absorption at any given wave



length. The ordinary method of using this instrument is to take a series of readings at different parts of the spectrum and thus gradually build up an absorption curve which will show any specific absorption bands present; but in this case the heads of the specific absorption bands had already been found, at  $614$  and  $530\mu\mu$  in the case of antimony trichloride. If, therefore, readings were taken every half-minute or so at these wave lengths alternately, with an occasional reading at intermediate wave lengths to check the fact that the points of maximum absorption remained constant, it appeared possible to obtain a quantitative record of the changes undergone by the chromogen.

Experiments were carried out, using a mixture of antimony trichloride with a stable cod-liver oil, and the results obtained are summarised in the curves in Fig. 3. It will be seen that these demonstrate a definite decrease in concentration of the chromogen responsible for the band at  $614\mu\mu$ , simultaneously with an increase in concentration of the chromogen responsible for the band at  $530\mu\mu$ .

#### DISCUSSION.

From this study of the colours attributed to vitamin A it appears that application of the "vitamin" reagents (arsenic or antimony trichloride) brings about a definite and characteristic series of changes in the chromogen molecule. The first conclusion to be drawn from this observation is the importance of the time effect in any work on the "vitamin" colours. In any attempt to estimate vitamin A colorimetrically, by measuring by means of the Lovibond tintometer the intensity of blue colour produced by these reagents, the time effect cannot be ignored. For, as was shown by Gibson and Harris, the Lovibond blue glasses against which these colours are compared have their maximal absorption between  $570$  and  $680\mu\mu$ , diminishing rapidly both above and below these limits. Therefore, while these glasses are suitable for measuring the initial colours produced by the "initial" bands at about  $587\mu\mu$  in the case of arsenic trichloride, or at about  $614\mu\mu$  in the case of antimony trichloride, they are obviously not suitable for measuring colours produced by the "second" bands at about  $475$  and  $530\mu\mu$  respectively. For the latter purpose red glasses, with maximal absorption between  $480$  and  $590\mu\mu$ , would be preferable. But, since absorption in this region of the spectrum may be due to other substances besides vitamin A, it is necessary to employ only the initial colours measured by the blue glasses in any application of the tintometer in the quantitative estimation of vitamin A. In the present study it has been shown that the initial bands rapidly lose their intensity, a significant difference being observed within a minute or so. Hence support is given to the suggestion first made by Willimott, Moore and Wokes [1926] that tintometer readings on "vitamin" colours be taken not more than 30 seconds after mixing.

Another conclusion which appears permissible is that the detection and estimation of vitamin A by colorimetric methods probably necessitates the

destruction of the vitamin. In the first paper of this series it was shown that application of condensing or oxidising agents to sterols or some of their derivatives might under certain conditions lead to their developing the property of giving with the "vitamin" reagents colours very similar in appearance to the "vitamin" colours. It may be possible that in the case both of the sterols and of vitamin A the chromogen responsible for the "vitamin" colours is not the physiologically active vitamin, but a condensation or oxidation product of it. The characterisation of these specific absorption bands is a necessary step in the examination of this question, and affords the opportunity of obtaining further information regarding the chemistry of the vitamin.

Finally, it must be remembered that these results have been obtained not on pure substances, but on oils and concentrates of complex composition, probably containing a number of different sterol derivatives. A direct connection has still to be proved between the vitamin and the chromogen responsible for the characteristic absorption bands here described. The blue colours attributed to vitamin A, and the absorption bands which have now been shown to characterise them, cannot be considered specific until they can be obtained from a definite chemical compound which possesses the physiological properties of the vitamin.

#### SUMMARY.

1. A spectroscopic study has been made of the colours produced by the "vitamin" reagents (arsenic and antimony trichlorides) on a series of cod-liver oils and concentrates whose vitamin A content had been ascertained by feeding tests.

2. In each case there have been discovered two absorption bands which appear to be characteristic of the chromogen. Arsenic trichloride gave bands at about 587 and  $475\mu\mu$ , and antimony trichloride gave bands at about 614 and  $530\mu\mu$ .

3. The chromogen on standing in contact with either reagent gradually passes from the stage giving the "initial" band (at about 587 or  $615\mu\mu$ ) to the stage giving the "second" band (at about 475 or  $530\mu\mu$ ). This change is accompanied by a gradual loss in blue colour and gain in red colour, which can be measured by means of the tintometer. In any study of the "vitamin" colours the time effect is therefore of great importance.

4. An explanation, based on these observations, is suggested of the discrepancy between the spectroscopic results previously obtained by Rosenheim and Drummond [1925], and those obtained by Euler, Myrbäck, Fink and Hellstrom [1927].

I desire to express my thanks to Prof. E. C. C. Baly for kindly affording me facilities for carrying out the spectroscopic work, to Dr R. A. Morton for advice and assistance, and to Mr P. W. Tainsh, chief chemist to Messrs Lever Bros., for a supply of essogen and cod-liver oil concentrate.

## REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.  
Euler, Myrbäck, Fink and Hellstrom (1927). *Z. physiol. Chem.* **168**, 11.  
Gibson and Harris (1927). *U.S. Bureau of Standards, Bulletin No.* 547.  
Prideaux (1926). *J. Soc. Chem. Ind.* **45**, 664.  
Rosenheim and Drummond (1925). *Biochem. J.* **19**, 753.  
Willimott, Moore and Wokes (1926). *Biochem. J.* **20**, 1292.  
Wokes and Barr (1927). *Pharm. J.* **i**, 758.  
Wokes and Willimott (1927). *Analyst*, **52**, 515.

# CXXV. THE ABSORPTION OF WATER BY GELATIN. PART II.

## THE NITRATE SYSTEM.

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*(Received June 11th, 1928.)*

In a previous paper [Jordan-Lloyd and Pleass, 1927] the absorption of water by gelatin in a system containing hydrogen, hydroxyl, sodium and chlorine ions was considered. The present paper deals with a system which is similar, except that nitrate ions are present in place of the chlorine ions. The experimental material and method were precisely the same as described in the previous paper. The ash content of the gelatin used in the present experiments varied from 0.02–0.01 %.

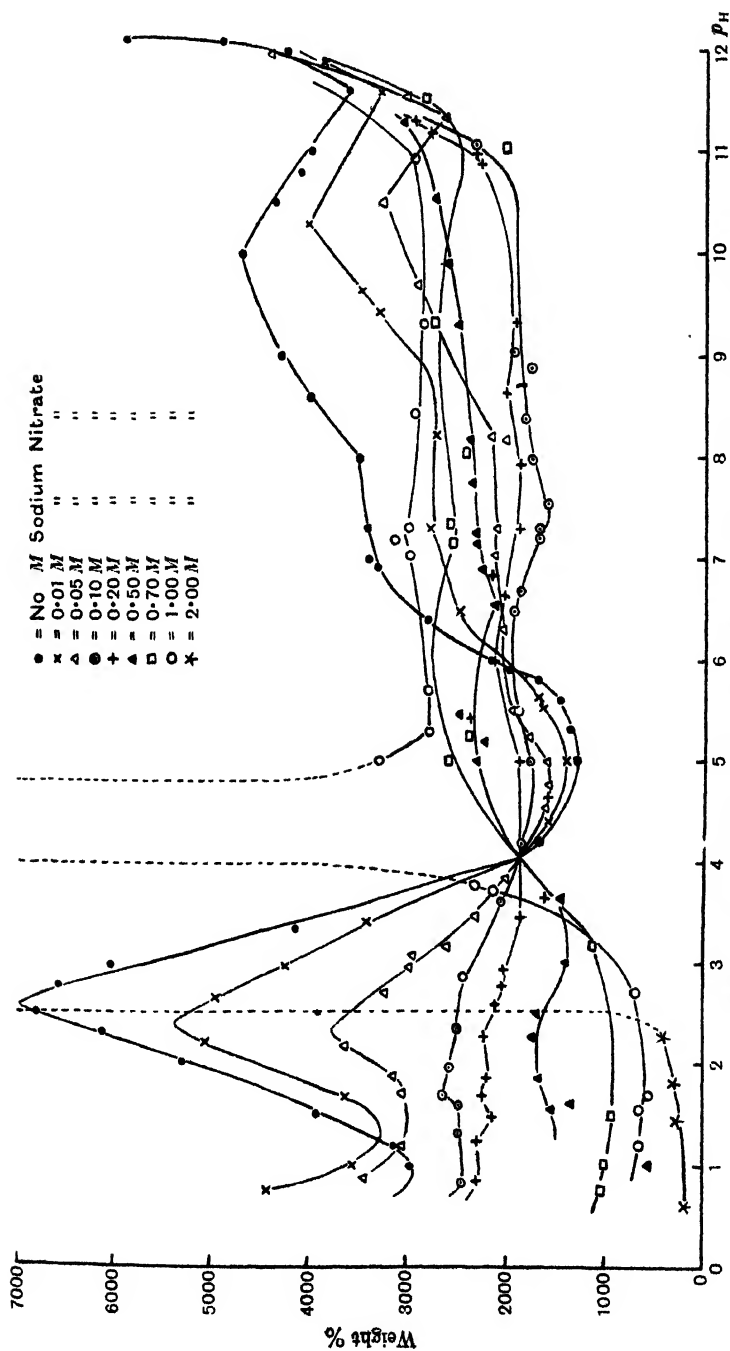
Three variables influencing water absorption were examined:  $p_H$ , temperature ( $t$ ) and concentration of sodium nitrate ( $M$ ). The experimental results are summarised in the curves shown in the figures.

The influence of  $p_H$  (nitric acid or sodium hydroxide) on the swelling of gelatin at 18° is shown in Fig. 1. In the absence of inorganic salts the swelling curve shows a minimum at  $p_H$  5.0, confirming the value previously found using hydrochloric acid. This value of  $p_H$  may therefore again be regarded as the isoelectric point. The curve shows a maximum in the acid range at  $p_H$  2.6. The  $p_H$  value at the peak of the acid swelling curve coincides with the value found for the maximum swelling in the hydrochloric acid system, but the degree of swelling is slightly greater. In the absence of inorganic salts the system on the alkaline side of the isoelectric point is identical with that described in the previous paper, *i.e.* there is a well-defined maximum of alkaline swelling at  $p_H$  10.0 and an additional broadly defined maximum between  $p_H$  7.5 and 7.9. At  $p_H$  values less than 1 or greater than 11.6, the gelatin dissolves in the external fluid.

It will be convenient again to consider separately the behaviour of gelatin in the four zones of hydrogen ion concentration centring respectively on  $p_H$  2.6 (acid zone);  $p_H$  10.0 (alkaline zone);  $p_H$  5.0 (isoelectric zone); and  $p_H$  7.0 (neutral zone).

### *The acid swelling zone ( $p_H$ 1–4).*

In the previous paper it was shown that in the absence of sodium chloride the swelling of gelatin in hydrochloric acid is in accordance with the predictions of the Proctor-Wilson equation [Procter, 1914; Procter and Wilson, 1916].



When sodium chloride is present, the repression of the swelling by the salt in concentrations less than 0.01 *M* occurs according to the predictions of Loeb's equation [Loeb, 1922]. In greater concentrations of sodium chloride, however, the repression of the swelling is considerably greater than the value calculated from this equation and coagulation of the gelatin may occur. In the nitrate system the form of the curves of swelling is similar to that of the corresponding curves for the chloride system, but certain differences are apparent. In the absence of mineral salts the degree of swelling in nitric acid is slightly greater than in hydrochloric acid at all values of  $p_H$ , the swollen weights at  $p_H$  2.6, for instance, being 7000 and 6500 % respectively on the dry weight of the gelatin. The addition of sodium nitrate to the nitric acid system causes greater repression of swelling than does the addition of an equivalent concentration of sodium chloride to the hydrochloric acid system at all salt concentrations from 0.01 to 1 *M*. Coagulation, defined as a reduction of the swelling of the protein below the value found at the isoelectric point (1300 %), which appears at 0.5 *M* in the acid chloride system, is first observed in nitric acid solutions at a nitrate concentration of 0.7 *M*. Coagulation can occur in the chloride system at all reactions below  $p_H$  4.0, in the nitrate system at  $p_H$  values less than 3.5. Coagulation, therefore, occurs less readily in the nitrate than in the chloride system, although in 2 *M* nitrate solutions, at  $p_H$  values less than 2.3, the degree of coagulation is greater than in the corresponding chloride systems, while at all  $p_H$  values greater than 2.3 the gelatin dissolves. The experimental curves showing the relationship between swelling and salt concentration in acid solutions (Fig. 7,  $p_H$  2.0, 2.5 and 3.0) show marked differences from the corresponding curves for the chloride system and only agree approximately with Loeb's theoretical curve when the salt content is low. At  $p_H$  2.0 the nitrate curve is similar in form to the chloride curve, suggesting that it represents a compound function, the sum of two (or possibly, in this case, three) simple functions of the salt concentration (*M*). When *M* is less than 0.2, the swelling curve, as in the chloride system, resembles in form the curve of Loeb's equation; when *M* is between 0.2 and 0.5 the curve is inflected and indicates that it may be turning to correspond to a negative linear function of  $\log M$ ; later, however, at concentrations of nitrate greater than 0.5 *M* there is a further slight inflection of the curve which suggests that the more concentrated salt solutions have not only a coagulating, but also a solvent action on the gelatin. The curve, therefore, illustrates the influence of three effects of the nitrate ion. The solvent action of solutions more concentrated than 0.1 *M* is shown more emphatically at  $p_H$  3.0 (Fig. 7). At this and higher values of  $p_H$  the solvent action completely masks any coagulating action.

Swelling in nitric acid is strongly influenced by temperature (Fig. 2). At temperatures below about 12° the temperature coefficient for the water absorption is not nearly as great as at higher temperatures. This may possibly be connected with the transformation of the gelatin from the gel form (gelatin A) to the sol form (gelatin B) [Smith, 1919], a change which begins at about

15°. In the absence of inorganic salts complete solution in the acid zone takes place at about 20° in nitric acid.

*The alkaline swelling zone ( $p_H$  8.0–11.5).*

The maximum gelatin swelling in solutions of sodium hydroxide free from salts occurs at  $p_H$  10.0 (Fig. 1). The effect of sodium chloride in the alkaline zone appears to be limited in salt concentrations up to 2 *M* to a repression of the swelling in accordance with the theoretical expectations of Loeb's equation. The effect of sodium nitrate in this zone shows some very marked differences

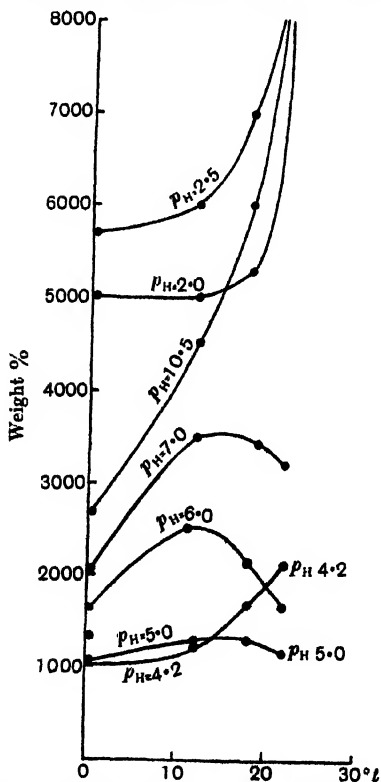


Fig. 2.  $t$  varying,  $M$  constant at zero,  $p_H$  constant at 2.0, 2.5, 4.2, 5.0, 6.0, 7.0 and 10.5.

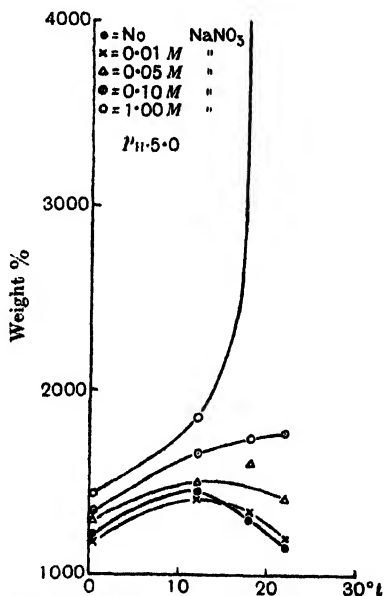


Fig. 3.  $t$  varying,  $p_H$  constant at 5.0,  $M$  constant at 5 values varying from zero to 1 *M*.

from the effect of sodium chloride. At 18° in concentrations less than 0.1 *M* the nitrate represses the alkaline swelling in a manner similar to the chloride, although for equimolecular concentrations of the two salts the repression is greater with nitrates than with chlorides, showing that even in dilute salt solutions the specific nature of the ions must be considered. At concentrations of nitrate greater than 0.1 *M* the dominant effect of the salt is to increase water absorption (see Fig. 7,  $p_H$  10.0). This undoubtedly arises from a solvent action. Complete solution at all  $p_H$  values in the alkaline zone occurs at 18° in all concentrations of nitrate greater than 1 *M* (Fig. 1).

*The isoelectric zone ( $p_H$  4-6).*

The position of minimum swelling in a system free from all salts is at  $p_H$  5.0. The presence of sodium nitrate in isoelectric or nearly isoelectric solutions causes increased absorption of water by gelatin, the amount absorbed at 18° being proportional to the logarithm of the salt concentration ( $M$ ), where  $M$  is less than 0.1 (Fig. 7,  $p_H$  5.0). At salt concentrations greater than 0.1  $M$  the water absorption is directly proportional to the salt concentration (Fig. 7,  $p_H$  5.0; and Fig. 8) until at concentrations of 1  $M$  when the gelatin is on the verge of solution. The effect of temperature on the action of the salt is very great. At 0° and 12° the water absorption is proportional to the logarithm of the concentration of the sodium nitrate at all concentrations examined (Fig. 5). At 18° and 22° the swelling increases rapidly with increase of salt concentration and complete solution takes place at 1  $M$  and 0.2  $M$  respectively. Nitrates, therefore, have a much greater dispersing action on gelatin B, which is present in the system only at temperatures above 15°, than on gelatin A. The effect of temperature on the absorption of water at different concentrations of nitrates is shown in Fig. 3. Below 12° and in concentrations of salt less than 0.1  $M$  the effect of the nitrates is similar to that of the chlorides and there is further a maximum of absorption of water at about 12° to 15°. A rise of temperature above 18° leads to a greater swelling and solvent action with chlorides than with nitrates.

*The neutral zone ( $p_H$  6-8).*

In this zone, in solutions of sodium hydroxide free from salts, swelling increases with increase of alkalinity, reaching a broadly defined maximum extending from  $p_H$  7.0 to 8.0 (Fig. 1). The effect of adding sodium nitrate in this zone is somewhat the same as the effect of adding sodium chloride, *i.e.* up to a concentration of nitrate of 0.1  $M$  the salt depresses the alkaline swelling; in concentrations greater than this, however, gradual increase in the nitrate concentration decreases the repression of the swelling (Fig. 7) until at a nitrate concentration of 1  $M$  at 18° swelling is very nearly the same as in the absence of all salts, but the gelatin is on the verge of solution. At 2  $M$  solution occurs in this as in all other zones. The solvent action of nitrates, which is the factor responsible for the secondary swelling in the more concentrated salt solutions, is very nearly the same in neutral, isoelectric and alkaline solutions (Figs. 7 and 8), and in all three zones it first makes itself apparent at a nitrate concentration of 0.1  $M$ . At 18° the solvent action in all three zones is greater for nitrates than for chlorides in equivalent concentrations.

The effect of temperatures at  $p_H$  7.3 and at different concentrations of nitrates is shown in Fig. 4. The curves closely resemble the corresponding curves for the chloride system. In concentrations of nitrate less than 0.1  $M$  they also resemble curves at  $p_H$  5.0 (Fig. 3) in showing a temperature of maximum swelling, which at  $p_H$  7.3 is at 18°. In solutions of 0.1 and 1  $M$  swelling increases rapidly with temperature until solution is reached. The



curves at all temperatures show definite minima of swelling which are much more strongly marked than in the chloride system. The salt concentration at the minimum of swelling in nitrates varies inversely with the temperature, minima being obtained at 0.20, 0.13, 0.10 and 0.04  $M$  at 0°, 12°, 18° and 22° respectively.

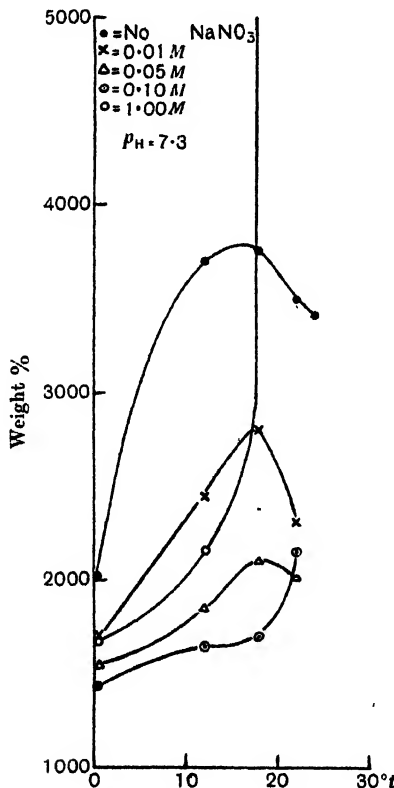


Fig. 4.  $t$  varying,  $p_H$  constant at 7.3,  $M$  constant at 5 values varying from zero to 1  $M$ .

#### DISCUSSION.

The behaviour of gelatin in the presence of nitric acid or nitrates presents at many points a strong contrast to its behaviour in the presence of hydrochloric acid or chlorides, although when considered from a physical-chemical viewpoint these systems are very similar, both nitric and hydrochloric acids being strong monobasic acids which combine with sodium hydroxide to produce readily soluble, highly ionised salts. The obvious difference in the two systems lies in the relative positions of chloride and nitrate in the Hofmeister series. "Hofmeister" effects are generally associated with fairly strong solutions of salts, and the contrast between the two systems is certainly most marked when the concentration of the salt is greater than 0.1  $M$ , although it is by no means limited to the higher concentrations of chlorides and nitrates. Nitric acid produces greater swelling than does hydrochloric acid and in the acid, neutral

and alkaline zones the depression of swelling caused by nitrates, even in dilute solution, is greater than that caused by chlorides at the same concentrations. Thus, even in dilute solutions, where the effect of the salts has been assumed to be entirely electrostatic, the divergence from a strictly quantitative relationship between the two systems suggests that the specific nature of the ions plays a part.

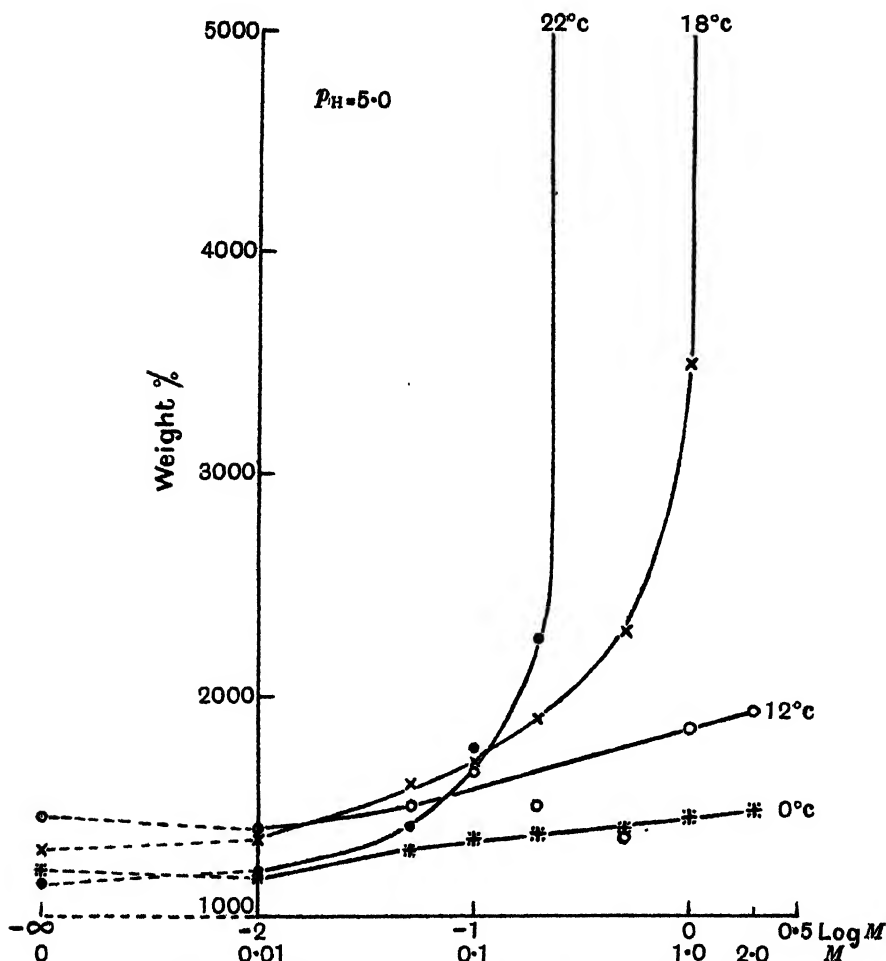


Fig. 5.  $M$  varying,  $p_H$  constant at 5.0,  $t$  constant at 0°, 12°, 18°, 22°.

It was shown in the earlier work on chlorides that the coagulation of gelatin by these salts depends on the  $p_H$  of the system. It will be seen on referring to Fig. 7 of the earlier paper [Jordan-Lloyd and Pleass, 1927] that at the position on the swelling-salt concentration curves where the swelling in acid solutions is equal to that in distilled water, there is a point of inflection which marks the beginning of coagulation. Coagulation may, therefore, be

given a numerical significance by defining it as the reduction of the water content of a gel below that amount which the isoelectric colloid would contain in equilibrium with distilled water. Chlorides coagulate gelatin at any  $p_H$  less than 4.0 if present in sufficient concentration. It can be seen that nitrates in acid solutions can also coagulate gelatin, the conditions of  $p_H$  and salt

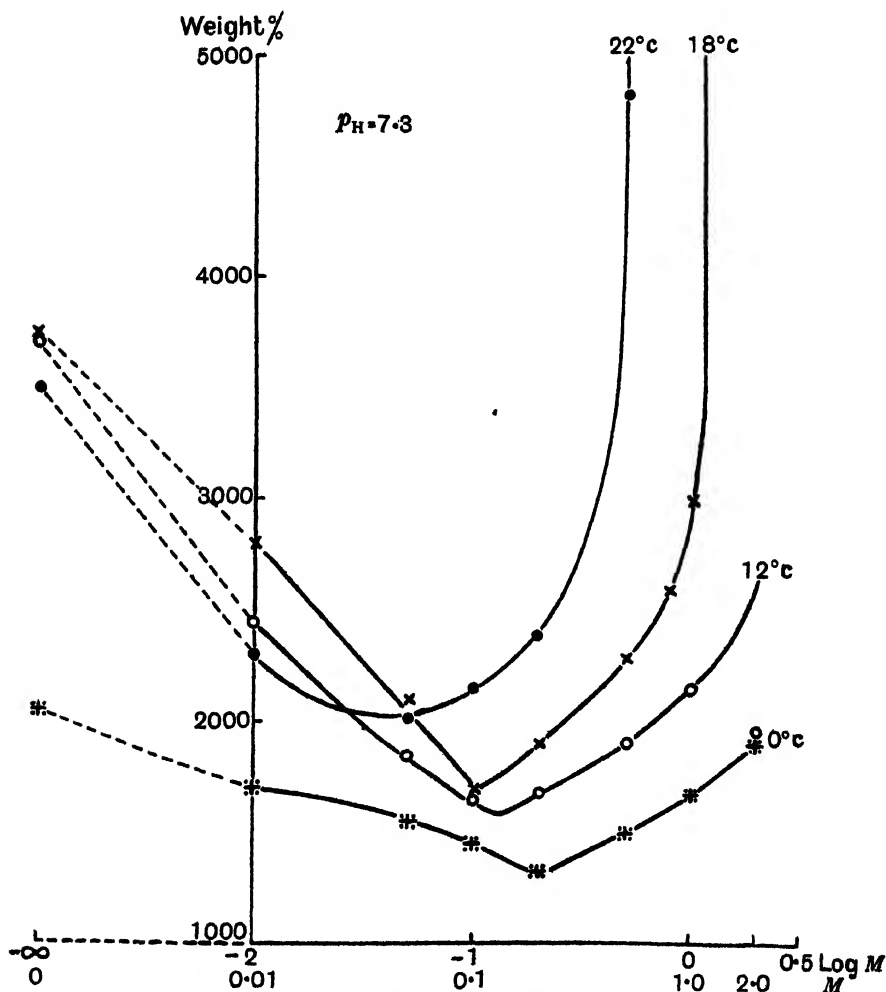


Fig. 6.  $M$  varying,  $p_H$  constant at 7.3,  $t$  constant at 0°, 12°, 18°, 22°.

concentration which can bring this about being more strictly defined than in the chloride systems, coagulation only occurring at  $p_H$  values less than 3.5 (Figs. 7 and 9). Fig. 9 shows clearly that there is a change in system at the coagulation point, which depends on the  $p_H$  of the system. At  $p_H$  values less than 3.0, nitrate concentrations greater than 0.6  $M$  (approx.) lead to coagulation.

At the corresponding point in the chloride system, 0.3  $M$  salt was sufficient

to start coagulation. The coagulating action of the nitrates only exists over a limited range of  $p_H$  and salt concentrations. Beyond these narrow limits excess of nitrate leads to solution of the gelatin. At 1  $M$  concentration nitrates dissolve gelatin over a  $p_H$  range from 4.0 to 5.0; at 2  $M$  over all  $p_H$  values greater than 2.3 (Fig. 7). At  $p_H$  4.1 the system is interesting—above 1  $M$  concentration of sodium nitrate the gelatin dissolves, below 0.7  $M$  the weight of the swollen gelatin is constant at a value of 1900 % on the dry weight, independent of the salt concentration. A similar point where water

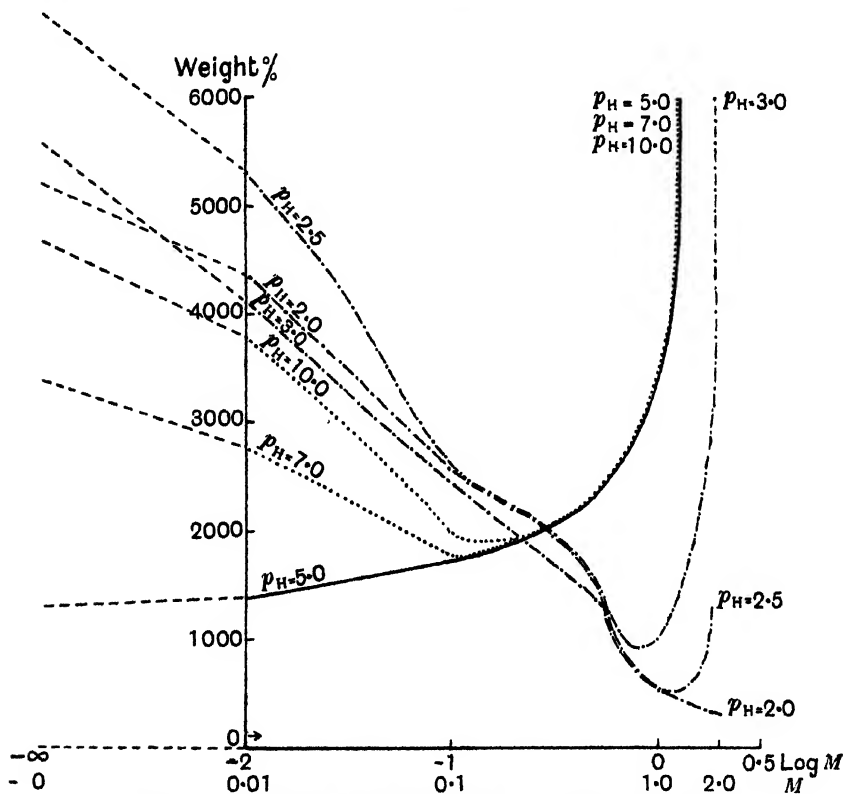


Fig. 7.  $M$  varying,  $t$  constant at 18°,  $p_H$  constant at 2.0, 2.5, 3.0, 5.0, 7.0 and 10.0.

absorption is independent of salt concentration is found at  $p_H$  4.4 in the chloride system, the swollen weight in this case being 1400. At  $p_H$  3.5, increasing the nitrate concentration from 0.6 to 1  $M$  leads to an increase of swelling followed by solution at higher concentrations. The effect of the nitrates at  $p_H$  3.5 is, therefore, similar to its effect in the neutral and alkaline zones (see Fig. 8). In these zones nitrates at a concentration of less than 0.1  $M$  depress swelling, at concentrations between 0.1  $M$  and 1  $M$  they lead to an increase in swelling proportional to the concentration of the salt. In the iso-electric zone sodium nitrate up to 0.1  $M$  resembles sodium chloride in leading

to an absorption of water which is proportional to the logarithm of the concentration of the salt, and which is, therefore, probably due to a similar mechanism, possibly adsorption. At concentrations greater than  $0.1\ M$  the effect of the addition of salt becomes identical with its effect in all other systems on the alkaline side of the isoelectric point (Fig. 7). The nitrates, therefore, have in solutions greater than  $0.1\ M$  an additional effect on the system which is either absent from the chloride systems or does not show itself in concentrations as weak as  $2\ M$  or less.

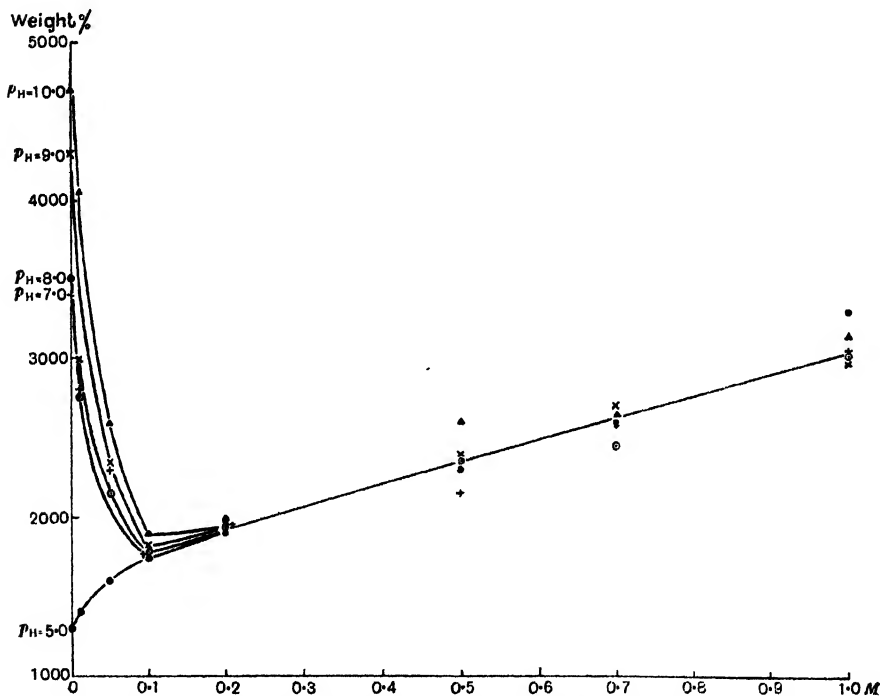


Fig. 8.  $M$  varying,  $t$  constant at  $18^\circ$ ,  $p_H$  constant at 5.0, 7.0, 8.0, 9.0 and 10.0.

In the previous paper it was pointed out that the mechanism of water absorption was very different in acid or alkaline swelling on the one hand, and in swelling under the action of salts on the other. The contrast between these two types is emphasised by the amount of swelling which can take place before the gelatin loses its property of cohesion. In acid swelling gelatin can absorb 70 times its own weight of water and still retain its cohesive properties and in alkaline swelling the capacity for holding water in a jelly phase is of the same order. In salt swelling, however, the capacity for holding water is only about half this value and solution in salt solutions appears to set in very rapidly once a critical water content has been passed.

## SUMMARY.

1. Sodium nitrate, up to a concentration of 0.6 *M*, in the presence of nitric acid suppresses the swelling of gelatin due to the acid. At greater concentrations its effect depends on the  $p_H$ . From  $p_H$  4.0 to 3.5 increasing salt concentration causes swelling; at  $p_H$  3.0 it causes coagulation of the gelatin; at 2 *M* coagulation occurs at all  $p_H$  values less than 2.3 but solution at all values greater than 2.3. At  $p_H$  4.1 over a range of 0.0–0.7 *M* nitrates have no effect on swelling; beyond this range they dissolve the gelatin.

2. Up to a concentration of 0.1 *M* sodium nitrate in the presence of sodium hydroxide suppresses the swelling of gelatin due to the alkali. At concentrations greater than this, increasing concentration of nitrate is accompanied by

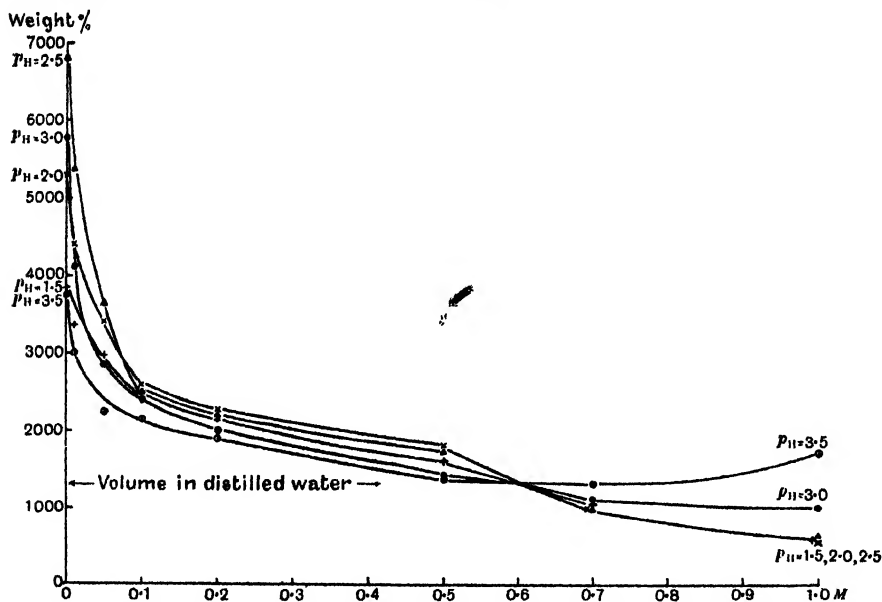


Fig. 9. *M* varying, *t* constant at 18°,  $p_H$  constant at 1.5, 2.0, 2.5, 3.0 and 3.5.

an increase in swelling, directly proportional to the concentration of the salt present. In solutions more concentrated than 0.1 *M* the gelatin is dissolved at 18°.

3. The swelling of gelatin due to nitric acid and sodium hydroxide increases as an exponential function of the temperature.

4. At the isoelectric point ( $p_H$  5.0), sodium nitrate promotes water absorption, swelling being proportional to the logarithm of the concentration of the salt up to 0.1 *M*. At greater concentrations swelling is directly proportional to the concentration of the nitrate.

5. Over a  $p_H$  range from 5 to 10 and at concentrations of salt greater than 0.1 *M*, swelling in solutions of sodium nitrate is influenced only by the salt concentration and is independent of the  $p_H$ .

6. At  $p_H$  5.0 a rise of temperature from  $0^\circ$  to  $12^\circ$  is accompanied by an increase in swelling which is the same in the absence of nitrate and at all concentrations of nitrate up to 1 *M*. In the absence of salt, swelling is at a maximum at  $12^\circ$ , in dilute solutions of nitrate at about  $15^\circ$  to  $18^\circ$ ; in stronger solutions there is no temperature of maximum swelling, the gelatin passing into solution as the temperature is increased. The temperature of solution is lower the greater the concentration of nitrate present.

7. At  $p_H$  7.0, in the absence of salt or in dilute solutions of nitrates, the effect of temperature on swelling is similar to its effect at  $p_H$  5. The temperature influences the position of minimum swelling at varying concentrations of nitrate, the salt concentration registered at this point diminishing with rising temperature.

#### REFERENCES.

- Jordan-Lloyd and Pleass (1927). *Biochem. J.* **21**, 1352.  
Loeb (1922). *Proteins and the theory of colloidal behaviour* (New York).  
Procter (1914). *J. Chem. Soc.* **105**, 313.  
Procter and Wilson (1916). *J. Chem. Soc.* **109**, 307.  
Smith (1919). *J. Amer. Chem. Soc.* **41**, 135.

## CXXVI. THE ASSAY OF VITAMIN A.

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*(Received July 2nd, 1928.)*

HUME and SMITH [1928] in a recent comprehensive survey of methods of assay of vitamin A have summarised the different kinds of response to different doses of vitamin A in animals previously depleted of this factor, as follows.

(a) Very small or no dose. No recovery.

(b) Small doses. Subnormal growth, graded quantitatively to the dose; later premature slackening.

(c) Larger doses. Normal growth for a time followed by premature slackening.

(d) Optimal dose. Normal growth to maturity.

Hume and Smith based this scheme partly on their own experience and partly on that of other workers. The writers have had this experience many times. But other types of response have also been encountered, a knowledge of which may eventually help in the elucidation of the rôle of the vitamin in the animal body. In tests on certain substances submitted to this laboratory for vitamin A assay, subnormal growth, graded quantitatively to the dose, has been obtained but instead of being followed by premature slackening the rate of growth has remained unaltered, and subnormal, for unusually long periods of time, some animals attaining maturity only after about 40 weeks on the experimental diet.

Another variation in response to equal doses of the same substance is also demonstrated. Animals which are apparently in a similar physiological or pathological condition, when considered ready for a curative test, have shown:

(a) immediate resumption of rapid growth;

(b) immediate resumption of subnormal growth;

or (c) a long latent period, often of several weeks, followed by a resumption of normal growth.

### *Technique.*

The technique of these experiments was that adopted throughout in these laboratories [Steenbock and Coward, 1927]. The sample of dried yeast used in the tests described in this paper was one which caused resumption of "normal" growth in a dose of 0.4 g. daily in a curative test on the vitamin B-free diet in use in this department. Hence its incorporation as 8% of the vitamin A-free diet makes it certain that vitamin B was not a limiting factor in these experiments.



*Examples of the continuous, subnormal response.*1. *An assay of vitamin A in a sample of cod-liver oil.*

The result obtained in the first 80 days of the curative period was practically in accordance with Hume and Smith's scheme, but the experiment was continued for a further period of 190 days for another purpose which will be reported later. The rat on the lowest dose of oil, 0.001 g., lived for a further 63 days and then died. The other three rats, still given their respective doses, maintained very slow growth for a further 90 days. They were then given a daily dose of 0.5 g. marmite dissolved in water in order to ascertain whether a shortage of vitamin B had possibly been the limiting factor in their growth. When this had shown no influence on their rate of growth for 30 days, it was

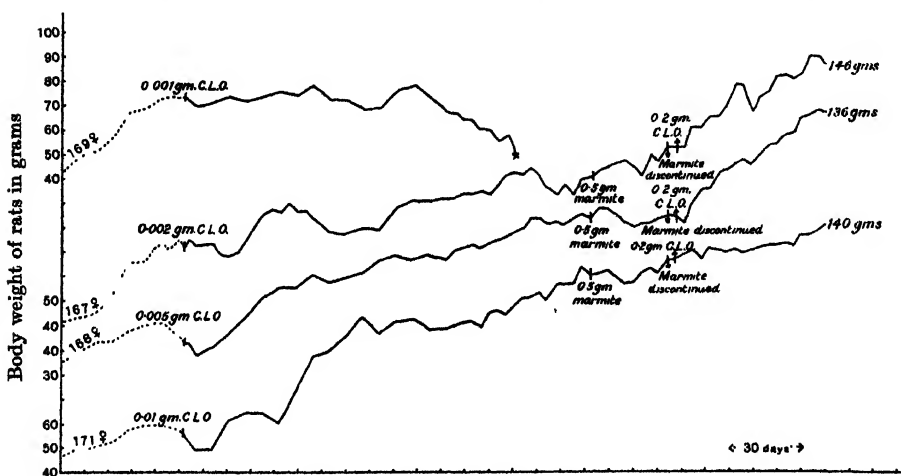


Fig. 1. Curves of growth of rats given different doses of cod-liver oil. The growth was graded to the doses during the first 80 days, then continued more uniformly nearly to maturity, the rat on the lowest dose, only, dying.

discontinued and 4 days later the daily ration of cod-liver oil was in each case increased to 0.2 g. The two lighter rats, 167 and 168, previously receiving the smaller doses of cod-liver oil, 0.002 and 0.005 g. respectively, responded to the higher dose in a very marked manner, growing to about 140 g., at which weight growth of does of this strain (pure bred albinos) tends to fall off somewhat. The fourth rat which had received the highest dose of cod-liver oil throughout the test (0.01 g.) was nearly mature when the dose was increased and merely maintained its former rate of growth. It must be accepted therefore that these are vitamin A curves throughout; for the behaviour on the highest dose of cod-liver oil proved that the rats had received ample supplies of vitamin B. (The lack of response to the extra vitamin B in the marmite does not prove this as the low supplies of vitamin A were evidently the limiting factor to growth at this point (Fig. 1).)

After the first more rapid response, the rats settled down to subnormal, steady rates of growth for a further 17 weeks with no premature slackening.

### 2. *An assay of vitamin A in a certain biscuit.*

The biscuit, finely powdered, was incorporated in the diets of the rats as 5, 10, 20 and 40 % respectively. Daily records of food intake were made and from them was calculated the total intake of the biscuit eaten by each rat per week. These figures are given in Table I as they are more instructive than the figures representing the percentages of the mixture incorporated in the diets.

Table I.

Rat	% mixture in diet	Total intake of vitamin mixture per week (g.)								
		1	2	3	4	5	6	7	8	9
M 216	5	1.8	1.9	2.0	1.8	2.4	2.4	2.0	2.6	2.9
259	"	1.9	1.8	2.5	2.2	2.5	2.8	3.4	3.4	—
330	"	2.2	2.5	2.2	2.6	2.6	2.8	2.5	3.7	—
212	"	1.8	2.4	1.7	2.3	2.3	2.3	3.0	2.8	—
211	10	5.0	5.6	6.7	6.9	7.8	7.8	7.8	8.6	7.9
258	"	3.6	4.0	4.6	4.6	5.8	5.5	4.9	6.1	6.5
254	"	2.9	3.1	3.8	4.1	4.7	4.7	3.9	4.0	4.3
326	"	4.3	4.6	4.7	4.9	5.4	6.2	6.4	—	—
327	20	7.6	9.8	9.8	10.6	10.4	12.6	15.2	—	—
214	40	14.8	15.2	18.0	19.2	20.0	20.0	19.2	25.0	22.4

(Other rats used in this assay gave the more general type of recovery curve and therefore are not quoted here.)

The rates of growth of these rats are nearly constant throughout the time of the experiments. They are subnormal, graded to the dose, and do not show any sign of premature slackening (Fig. 2).

### 3. *An assay of vitamin A in the general London milk supply of November and December, 1927.*

Three bucks of one litter used in this test gave subnormal growth, graded to the dose, without premature slackening. The curves of growth are shown in Fig. 3.

### 4. *An assay of vitamin A in a certain mixture submitted to this department for analysis.*

Three bucks of the same litter as those in the last experiment were used in this test and again gave subnormal growth, graded to the dose, without premature slackening. The curves of growth are shown in Fig. 4. The mixture was incorporated in the diets as 2, 5 and 20 % respectively. Daily records of food intake were made and the amounts of the mixture eaten per week were calculated. The figures are summarised below.

Rat	% mixture in diet	Total amount of mixture eaten per rat per week (g.)						
		1	2	3	4	5	6	7
G.R.L. 32 A	2	0.92	0.94	0.94	0.94	0.94	1.4	1.4
" C	5	2.0	1.95	1.95	2.1	2.1	2.25	2.5
" E	20	9.0	9.6	9.8	9.2	11.4	11.6	14.8

#### *Varied types of response to the same dose.*

Three different types of response to a given dose of a substance containing vitamin A are frequently met. They may be described as (a) immediate resumption of rapid growth which may be continued to maturity, (b)

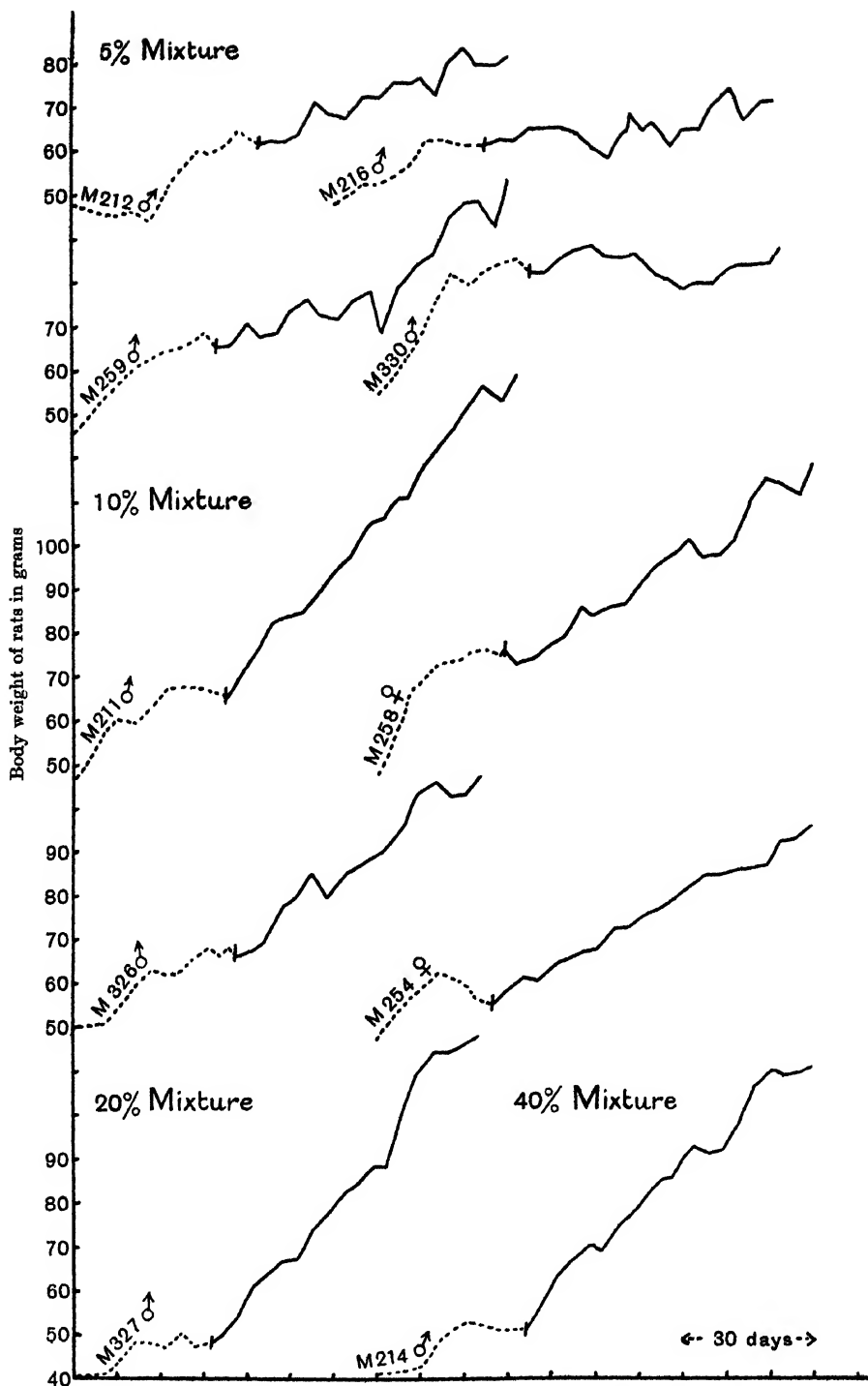


Fig. 2. Growth of rats, graded to the dose of vitamin A, without premature slackening. A more accurate comparison is obtained by comparing these curves with the weekly records of intake of the vitamin mixture rather than with the percentage incorporated in the diet.

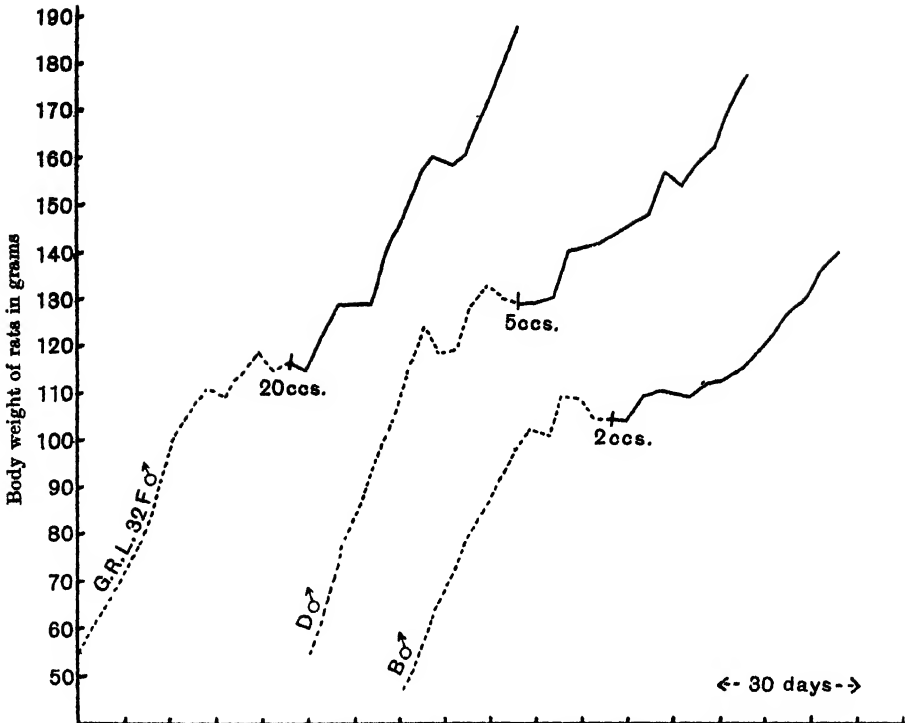


Fig. 3. Growth of rats, graded to the dose of London milk. No premature slackening.

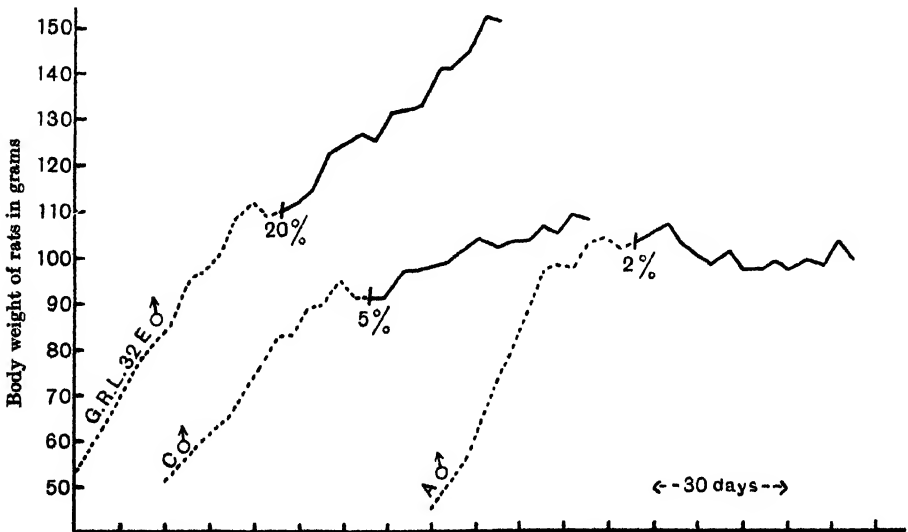


Fig. 4. Growth of rats, graded to the dose of vitamin A. The weekly intake corresponds with the percentage of the material in the diet in this experiment.

immediate resumption of growth but at a lower rate than (a), (c) a long steady period followed by rapid resumption of growth. In the last, presumably, growth is suspended until possible pathological conditions are cleared up. Curves showing the variations in response to a given dose are given in Fig. 5. The variability of the response is discussed here because it is obviously of the greatest importance to give a rat the chance of recovering from disorders before deciding that a particular dose of a substance is inadequate in vitamin A. It must be admitted, however, that this cannot be the whole explanation, for far more uniform and comparable results are obtained when rats from one litter are used for a comparison than when a miscellaneous selection of unknown history are used, even though, from a consideration of their growth curves, they may appear to be in a similar condition.

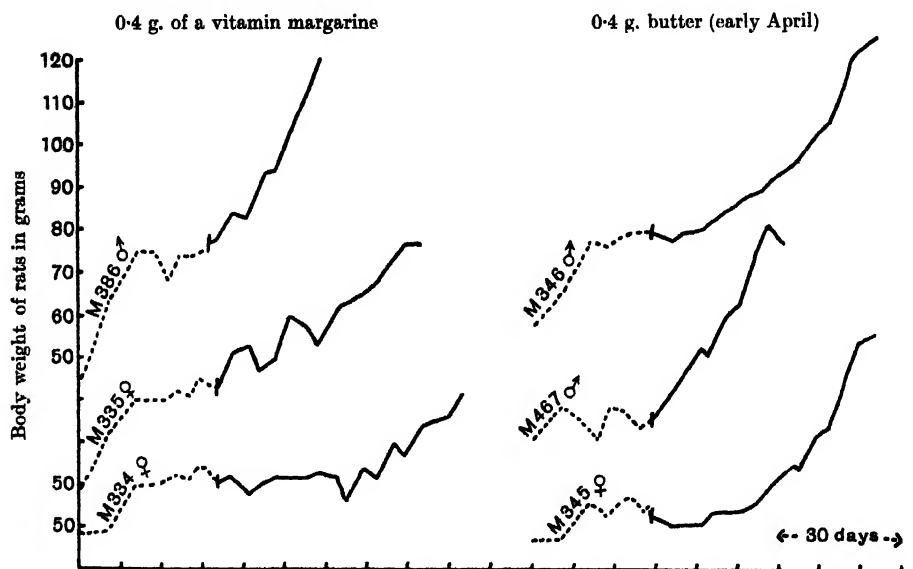


Fig. 5. Two examples of variation in response to equal doses of the same substance. Rats M 386, 467 show immediate resumption of rapid growth; rats 335 and 346 show immediate resumption of subnormal growth; rats 334 and 345 show a long latent period followed by resumption of rapid growth.

For an assay of the vitamin A content of a substance in this laboratory three or four rats are used for testing each of several different doses, the lowest of which is expected to be too small for resumption of growth, and the highest probably greater than is necessary for normal growth. A comparison of the whole series of results with those obtained from a similar series on the substance with which the unknown is to be compared, gives the desired information as to the vitamin A content of the test material. When rats from our own colony are used, the comparison can be made with about half the number of animals.

## SUMMARY.

A type of response to small doses of vitamin A is described. It is a resumption of growth, after the animal has become steady in weight on a deficiency of this factor, which is subnormal in rate, graded to the dose and without premature slackening.

The importance of a long test period, of eight or more weeks, is emphasised.

## REFERENCES.

Hume and Smith (1928). *Biochem. J.* **22**, 504.

Steenbock and Coward (1927). *J. Biol. Chem.* **72**, 765.

## CXXVII. URICASE AND ITS ACTION.

### PART I. PREPARATION.

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*(Received May 21st, 1928.)*

Two main tendencies may be distinguished in the development of modern enzymology. One of these consists in the chemical analysis of the enzyme and the other in the study of the action of the enzyme under natural physiological conditions.

The consensus of opinion now is that an enzyme must possess two characteristic properties—a definite physico-chemical state, whose change involves changes in the activity of the enzyme, and an active and specific group or centre.

In the present state of our knowledge we cannot yet state in what depends the specificity of an enzyme, except that no doubt exists that the colloidal carriers and active centres may be very different. It may be as a result of the specific binding with the substrate that a given enzyme acts upon any particular substance. As to the active groups it would appear that even in one group of enzymes, such as, for example, the oxidases, there are purely organic groups as well as others containing some metal, which is not in every case the same. In some cases this is iron, whilst in others, *e.g.* uricase, copper is present, as will be shown in Part III of this series.

It appears to us that the researches of Warburg [1928], as well as the application of micromethods to purified enzyme preparations, will, at least for the oxidases, allow the nature of this active group to be to a considerable extent elucidated.

It is not known whether only one or several active groups are present, or whether these are identical; this applying in particular to such complex enzymes as succinoxidase. This problem is of the utmost importance for those enzymes which influence reversible reactions of synthesis from two different substrates. Not only the carrier, but also the active group, may possess a specific character. Further research upon the structure of enzymes should therefore take both these factors into account, analysing each separately.

At the present stage of development of biochemistry it would appear that the study of the active group is likely to yield more fruitful results than the study of the colloidal carrier, in particular in those cases where the active

group contains some metal. As for the carrier, comparative research might be carried out upon it, such as the effect of changing  $p_H$  and temperature, and of inhibitory agents upon the action of the same enzyme derived from different organisms.

Certain of the features possessed by the carrier might, however, be elucidated at the present juncture, such as whether or not the same enzyme derived from different animals or plants possesses the same colloidal carrier. Such investigations should be carried out upon an enzyme acting specifically only upon one compound, in order to exclude as far as possible the danger of working upon mixtures of several enzymes, or upon different enzymes of closely related but not identical functions.

Such experiments might include determinations of the velocity of diffusion through various membranes, of solubility, of the action of temperature, of the precipitating effect of various salts, etc., as well as possibly the effect of changing  $p_H$  and the investigation of phenomena of cataphoresis, although here the active group also may be involved.

Similar research would also be desirable for the investigation of the active group. It is possible that several groups having a similar function might act in a similar or even identical manner upon a substrate, whence it might appear that two enzymes are identical, although both their active groups and their colloidal carriers may be different. This is all the more important and interesting in that the same substrate may be decomposed by a given organism under the influence of various catalytic factors.

Due consideration should, of course, be given to the purely energetic aspect of oxidations catalysed by various substances, *i.e.* to the relationship between energy absorbed during an endothermic reaction and evolved during a coincident exothermic reaction. This factor, whilst of first-rate, and for some reactions of oxidation decisive, importance, is not the only one concerned—questions of chemical affinity also play an important rôle. A number of other factors might, however, be involved, entirely distinct from the question of affinity for oxygen.

The first of these is adsorption. A second factor is that of the possibility of some chemical change occurring in the substrate under the influence of specific adsorption. It is quite probable that the introduction of certain compounds leads to such changes in the structure of the substrate as would facilitate its combination with oxygen under the influence of certain chemically-active groups which are, however, distinct from those directly responsible for oxidation.

Such sensitisation might, however, also be brought about by the agency of ordinary non-specific adsorption, and in view of the possibility of the existence of different types of adsorption of the same substance upon different adsorbents, the same active group may act differently according to the circumstances.

The specific action of the various oxidases may therefore depend upon a number of factors. These may be due not only to fundamental differences in



the active group but equally well to the concentration of the substrate within the close vicinity of peroxides, and to changes in the substrate brought about by adsorption or by supplementary groups of the adsorbent, distinct from those responsible for transference of oxygen or activation of hydrogen. These factors are all functions of the carrier, and support the view that both the carrier and its active group are concerned in the specific action of a given enzyme.

The carrier may be supposed to play a more or less important rôle in producing chemical change of the substrate, in accordance with the nature of the action of the given ferment, but the necessity of the existence of some spatial configuration favourable to oxidation must be emphasised. This consideration applies equally to reactions of synthesis, in which the combination of two different substances depends upon the active groups being so placed that the substrates are able to come into contact [cf. Euler, 1926].

Many of the above problems are at present capable of solution. In the research of which this is the first communication they will be considered, using as experimental material uricase, a widely distributed and unusually specific ferment, acting exclusively upon uric acid.

The experimental programme in view was first to prepare as pure as possible samples of uricase from different sources, such as bovine kidneys, frogs and wheat seeds, and to compare the carriers and the active groups of these preparations with a view to establishing ultimately their identity or otherwise. Before commencing these investigations, however, it was necessary to establish whether uricase is a dehydrogenase or a true oxidase.

At the same time work is proceeding in this laboratory upon the oxidation or decomposition of uric acid through the agency of a biocatalyst, and of various purely chemical catalysts possessing an oxidising or dehydrogenating influence, as it is our opinion that only a very wide programme of research will here lead to the elucidation of the rôles of the colloidal carrier and the active group.

## EXPERIMENTAL.

### I. *The purification of frog uricase.*

The enzyme was purified by precipitation with acetone, alcohol or ammonium sulphate, or by dialysis.

The precipitation by means of acetone was carried out according to Przylecki [1925]. 300–500 g. live weight of frogs were comminuted in a mincing-machine and the hash was left for 48 hours in a mixture containing 50 g. of chloroform, 100 g. of glycerol and 500 cc. of water. Water was then added in amount equal to ten times the weight of tissue taken and the mixture left for 24 hours with occasional shaking, after which it was filtered through cotton-wool.

Two volumes of acetone were added to one of the extract and the flocculent precipitate was filtered after 30–60 minutes through a fluted filter-paper and

allowed to dry on the paper. The filtrate contained no uricase, which was present in its entirety in the residue.

Uricase may be precipitated by acetone and left in contact with the latter for several days and washed, after filtering, with 10–20 litres of 65 % acetone without any impairment of its activity, as is shown by the results given in Table I. The apparent diminution in the yields of uricase previously reported by the author was due, not to any deleterious effect of acetone upon the enzyme, but to its incomplete removal from the precipitate before solution in water or physiological saline solution. As a result of this, acetone was present in the reaction mixture and able to exert its inhibitory influence, possessed in common with alcohols, upon the reaction of uricolysis. This inhibitory action is, as will be shown later, reversible under certain conditions.

Table I. *mg. of uric acid found in 1 cc. of solution consisting of equal volumes of sodium urate solution at  $p_H$  7.5 and of acetone-precipitated uricase.*

Serial no. of prepara- tion	Uric acid at beginning of ex- periment	Material used as source of uricase	After 12 hours' action of extract of acetone precipitate								
			Residue left on evaporation of acetone filtrate	Acetone precipitate washed with 30 litres of acetone	Time in hours intervening between addition of acetone and filtration						
					$\frac{1}{2}$	1	6	12	24	51	72
5	0.44	Bovine kidneys	0.43	0.09	0.09	0.08	0.09	0.08	0.08	0.09	0.09
7	0.71	Bovine kidneys	0.71	0.10	0.12	0.11	0.11	0.11	0.13	0.11	0.12
10	0.34	Wheat seed	0.35	0.07	0.08	0.07	0.07	0.06	0.08	0.08	0.06
12	0.50	Wheat seed	0.50	0.13	0.13	0.14	0.13	0.15	0.13	0.14	0.13
14	0.43	Frogs	0.42	0.09	0.10	0.09	0.10	0.10	0.10	0.09	0.10
15	0.65	Frogs	0.64	0.13	0.12	0.12	0.12	0.13	0.13	0.12	0.13

The filter-paper coated with the dried residue was cut into narrow strips and extracted by prolonged shaking with water or 1 % salt solution. Where larger masses of residue, hardened by keeping, were to be dissolved, they were first powdered in a mortar before extraction. The extract was filtered after 24–26 hours, when a clear filtrate was obtained, giving flocks of coagulated protein upon boiling. The amount of precipitated protein so obtained was small in comparison with the volume of filtrate, and it was noticed that this was especially so for those extracts prepared from uricase which had been left for over a day after precipitation in contact with acetone.

A comparison of the uricolytic power of the glycerol extract and of the second extract, at equivalent dilutions (*i.e.* the residue obtained after adding 2 litres of acetone to 1 litre of glycerol extract is dissolved in 1 litre of 1 % salt solution or water), shows that in most cases that of the original extract was less than that of the purified product. The values found are given in Table II.

Table II. *Frog uricase. Decomposition of uric acid under the influence of uricase from the same glycerol extract purified by different methods. Uricolytic action is expressed in percentages of the original quantity of uric acid decomposed in 10-16 hours at 37°.*

In most cases the successive precipitates from a litre of glycerol extract were extracted with an equal volume of water or saline solution, except in the case of dialysed enzyme preparations, where the acetone residuc extract was made up to a litre and poured into the dialysers, which were surrounded by a litre of water. In all cases equal volumes of uricase and of urate solutions at a constant concentration (this was 50-100 mg. per 100 c.c.) were taken.

Serial number of preparation	Crude glycerol extract	Extract purified by			
		acetone	acetone and dialysis	acetone and alcohol	acetone, ammonium sulphate, alcohol
4	30	49	47	38	29
6	50	83	91	49	38
11	58	90	99	53	50
13	45	78	84	41	36
16	54	81	80	52	43

This enhanced activity of uricase after precipitation with acetone or alcohol has been noticed by Wiechowski and Wiener [1907] and by Batelli and Stern [1909]. This may be due to various causes. In the first place it would appear that uricase is exclusively in adsorption both in the cell and in the glycerol extract. This view is supported by the absence of this ferment in the free state from mammalian blood. Acetone precipitation has the effect of irreversibly coagulating many of the colloidal constituents of the cell, from which free uricase may then be extracted. It is further possible that concentrated acetone solutions elute uricase from the colloids precipitated by this agent, and that the latter are at the same time so denaturated that uricase is no longer adsorbed upon them, even after the removal of the acetone. The fact that acetone-purified uricase is easily dialysable, whilst that of the crude extract or of the tissue juices does not diffuse through a similar membrane, or at least does so very slowly, would appear to support this explanation.

Adsorbed uricase may act more feebly than unadsorbed for several obvious reasons.

A number of observations indicate that intracellularly uricase is present almost exclusively in the adsorbed state. This is shown by the difficulty with which it can be extracted and by the fact that a large excess of chloroform has to be employed for its quantitative extraction. Wiechowski and Wiener found that extraction was more complete in a slightly alkaline medium, and this we have been able to confirm.

The greater activity possessed by extracts of the acetone precipitate may also be due to the separation of the enzyme from inhibitory substances which are probably present in the glycerol extract.

Uricase purified by one acetone precipitation still contains a comparatively large quantity of impurities which are able to reduce methylene blue. Thus 10 cc. of the second extract without the addition of uric acid are able in 24-36 hours to decolorise an equal volume of 0.1 % methylene blue solution.

Uricase was further purified by dialysis or ultra-filtration, as a second acetone precipitation gave very poor yields of enzyme unless much greater quantities of acetone than were used in the first precipitation were added. The best results were given by dialysis, filtrates from Berkefeld candles or certain ultrafilters being less pure. Dialysis was carried out by placing uricase solution in paper dialysing tubes (No. 579, manufactured by Schleicher and Schüll), which were kept immersed in water or 1 % sodium chloride solution for 3 days, with the addition of 1 % thymol solution to prevent infection. The dialysate so obtained possessed a very strong uricolytic action, whilst at the same time it was almost free of substances reducing methylene blue. Such dialysates had often a greater uricolytic action than their parent preparations of uricase, in spite of the dilution being doubled. The results obtained are given in Table II.

The dialysates were clear, colourless liquids, which on boiling with sodium chloride gave only an insignificant opalescence. They were from 1000 to 3000 times as active as the frog tissues from which they were originally derived, *i.e.* 1 g. of dry dialysed uricase achieved decomposition of a quantity of uric acid 2000 times greater than that decomposed within the same time by 1 g. of the original tissue (cf. Table III), using in both cases different volumes of extract with the same volume of uric acid solution. A very similar degree of purity was obtained by using fairly large pore ultrafilters or collodion membranes supported on sintered glass. The filtrates thus obtained were also powerfully uricolytic, and barely gave traces of precipitate on boiling.

A third method applied to the purification of uricase consists in reprecipitating a solution of the first acetone residue with ethyl alcohol, or with a mixture of ethyl alcohol with acetone. The addition of these reagents in inverse order gave not only a lower yield of uricase but also a more impure product.

The acetone precipitate was dissolved in 1 % sodium chloride solution or water and 5–10 volumes of 96 % alcohol were added, the mixture was well agitated, and left for 15–30 minutes at a temperature of 0°, after which it was poured into a fluted filter paper. The residue was dried as quickly as possible, using an electric fan. The white powder so obtained rapidly darkened on exposure. Such a preparation of uricase may be kept apparently indefinitely in a stoppered bottle without loss of activity. The powder is extracted with water or 1 % sodium chloride solution when required and filtered, most of the impurities still present remaining on the filter-paper, whilst the filtrate is colourless, does not foam on shaking, is not opalescent, and forms scarcely any precipitate on boiling.

Further purification was effected in the following way. Twenty volumes of alcohol were added to the filtrate when a white precipitate appeared, which was filtered off after decantation of most of the supernatant fluid. The residue was washed with 95 % alcohol at 0° when a white residue remained, which in the same way as the previous preparation darkened on exposure to the atmosphere.

The product of double precipitation with acetone and alcohol successively yielded on dialysis an exceptionally pure product, although the yield of active substance was considerably less than that obtained by dialysis after acetone precipitation alone. The former dialysate contains an infinitesimal solid content, coupled with an unusually intense uricolytic action.

The fifth method applied was to salt out the aqueous extract of the first acetone residue with concentrated ammonium sulphate and to precipitate the extract of the residue with ten times its volume of alcohol, the further procedure being the same as for ordinary alcohol precipitation. This method also yields preparations of great activity calculated on unit weight of dry enzyme. The results are given in Tables II and III. Uric acid was in all cases determined by the colorimetric method of Folin and Denis.

Table III. *Estimation of the degree of activity of frog uricase at different degrees of purity.*

(Mean values in heavy type.)

Type of preparation	Solid content (mg. per litre)	Mg. uric acid decomposed by 1 g. in 1 hour*	Ratio of activity of 1 g. of given pre- paration to that of 1 g. of tissue
Tissue pulp (calculated on glycerol extract)	—	7.0, 8.7, 6.5, 9.1, <b>7.82</b>	—
Dialysed acetone precipitate extract	83.2, 67.0, 43.1, 35.0	10,800, 15,400, 20,900, 26,000, <b>15,700</b>	2010
Filtered dialysate of acetone precipitate extract	31.0, 24.0, 29.1, 19.5†	15,500, 23,700, 17,800, 38,150, <b>26,300</b>	3370†
Dialysate of extract of product obtained by successive precipitation with acetone, ammonium sulphate and alcohol	23.1, 17.6†, 23.4	13,200, 19,300, 11,600, <b>14,700</b>	1884

\* Calculated from 12 hours' action.

† 4240 maximum.

## II. *Purification of uricase derived from mammalian kidney.*

It has been shown by Batelli and Stern [1909] and Wiechowski and Wiener [1907] that uricase from mammalian kidneys may be purified by acetone or alcohol precipitation, or by the elimination by dialysis of certain dialysable constituents of the tissues (uricase does not pass through the membrane under these conditions). The use of combinations of these methods, which were not applied by these workers, shows that very concentrated preparations of uricase may be obtained both by double precipitation and by precipitation and dialysis.

The chief factor for the preparation of powerfully uricolytic products from mammalian organs is, as for frogs, the addition of sufficiently strong solutions of glycerol and chloroform to the finely comminuted tissue. Usually the following procedure was applied. To 100 g. of well-minced tissue 25 cc. of chloroform, 75 cc. of glycerol and 100 cc. of water were added, and the mixture was left for 48 hours with frequent shaking. A litre of water was then added,

and the whole left a further 24 hours with occasional shaking, after which the mixture was filtered through cotton-wool or muslin. The uricolytic power of the extract so obtained differs little from that found by Batelli and Stern, also for bovine kidneys, namely, 100 g. of tissue decomposed in 1 hour 1.06 g. of uric acid, whilst our extract of 100 g. of tissue decomposed 0.98 g. in the same time. It would hence appear that the uricase present in the tissues can be almost quantitatively eluted from them.

The same measures of purification were carried out on the above extract as for that from frogs—the results are given in Tables IV and V.

Table IV. *Bovine kidney uricase.*

(Procedure as in explanation to Table II.)

Serial number of preparation	Crude glycerol extract	Extract purified by			
		acetone	acetone and dialysis	acetone and alcohol	acetone, ammonium sulphate, alcohol
23	70	89	91	64	55
24	53	85	90	50	42
25	47	70	85	39	39
26	63	82	87	51	42

Table V. *Estimation of the degree of activity of bovine kidney uricase at different degrees of purity.*

(Mean values in heavy type.)

Type of preparation	Solid content (mg. per litre)	Mg. uric acid decomposed by 1 g. in 1 hour*	Ratio of activity of 1 g. of given preparation to that of 1 g. of tissue
Tissue pulp (calculated on glycerol extract)	—	12.0, 13.1, 11.3, <b>12.2</b>	—
Acetone precipitate extract	74, 65.1, 49.3	15,450, 14,950, 25,000, <b>18,500</b>	1512
Dialysed acetone precipitate extract	38	26,700	2220
Filtered dialysate of acetone precipitate extract	39.4, 26.9, 31.7	24,140, 31,470, 32,000, <b>28,900</b>	2370
Dialysate of extract of product obtained by successive precipitation with acetone, ammonium sulphate and alcohol	30.3, 24.0, 19.4	21,800, 24,100, 25,100, <b>23,700</b>	1943

\* Calculated from 12 hours' action.

### III. *Purification of uricase derived from wheat seed.*

The same treatment was applied to wheat seed as to frog or kidney tissue, and the results obtained show that the same methods are applicable to the preparation and purification of wheat uricase as in the two former cases. The acetone precipitate can similarly be dialysed, yielding an extremely pure product. The results obtained are given in Table VI.

Table VI. *Wheat seed uricase.*

(Procedure as in explanation to Table II.)

Serial number of preparation	Crude glycerol extract	Extract purified by		
		acetone	acetone and dialysis	acetone and alcohol
32	35	59	65	41
34	51	71	—	—
35	57	66	76	—
37	43	74	92	—

## REFERENCES.

- Batelli and Stern (1909). *Biochem. Z.* **19**, 219.  
 Euler (1926). *Chemie der Enzyme* (München).  
 Przylecki (1925). *Arch. Internat. Physiol.* **24**, 237.  
 Warburg (1923). *Katalytische Wirkungen der lebendigen Substanz* (Berlin).  
 Wiochowski and Wiener (1907). *Hofmeister's Beiträge*, **9**, 247.

# CXXVIII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS. PART III.

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(Received May 30th, 1928.)

THE work described in Parts I and II [Butcher, Pentelow and Woodley, 1927] has been continued and the present paper includes the following experiments over periods of 24 hours:

<i>R. Lark</i>	Series VI	September 7-8	Table XIII	Fig. 11
	Series VII	September 22-23	Table XIV	Fig. 12
	Series VIII	October 12-13	Table XV	Fig. 13
	Series IX	November 2	Table XVI	—
	Series X	November 17-18	Table XVII	Fig. 14
<i>R. Itchen</i>	Series V	August 23-24	Table XVIII	Fig. 15
	Series VI	September 30-Oct 1	Table XIX	Fig. 16
	Series VII	November 10-11	Table XX	Fig. 17
	Series VIII	December 14-15	Table XXI	Fig. 18

## *Variation in dissolved oxygen.*

*River Lark. September-November.* The chief characteristic of the present series of observations on the River Lark is the markedly lower values obtained for the maximum and minimum values of oxygen saturation compared with those of the spring and summer. At this period of the year, the absence of diatoms, together with a heavy cutting of weeds in September and the death of most of the green plants in October, has resulted in a decreased production of oxygen; the consumption of oxygen by decomposing plants has continued, and consequently the maximum and minimum values of oxygen saturation for these months are lower than at any previous time.

It would appear that the main factors governing the oxygen content of a river of this character are:

- (1) the quantity and type of plant life present, these factors being governed by the season of the year;
- (2) the prevailing actinic conditions.



The relative effect of these factors is seen below.

(1) The combined effect of the quantity and type of plant life present together with that due to prevailing actinic conditions is seen from the values of the oxygen maxima and minima throughout the year.

*Percentage saturation of dissolved oxygen.*

	Maximum	Minimum	
March 30-31	157	65	
April 27-28	178	57	
May 18-19	181	36	
June 28-29	111	37	
August 4-5	124	42	
September 7-8	71	39	
September 22-23	70	26	
October 12-13	49	27	Abnormal results due to pollution by beet-sugar factory effluents
November 17-18	10	2	

(2) Since the plants do not vary from day to day, a study of the oxygen values determined at the same hour on two successive days gives the effect of the day-to-day difference in actinic conditions.

	Date	Time	% saturation	Temp. ° C.	Remarks
R. Lark	March 30	7 p.m.	119	9.4	Fine
	" 31	7 "	116	9.25	Dull
R. Itchen	April 6	9 "	90.5	9.4	"
	" 7	9 "	81	7.0	Clear after dull day
R. Lark	" 27	6 "	137	11.0	Bright
	" 28	6 "	163	11.5	After bright day
R. Itchen	May 4	6 "	101	11.6	Dull
	" 5	6 "	115	13.6	Sunshine
R. Lark	" 18	6 "	165	15.0	Bright
	" 19	6 "	177	15.2	"
R. Itchen	" 31	10 a.m.	97	10.6	Dull
	June 1	10 "	104	11.9	"
R. Lark	" 28	10 "	57	12.6	"
	" 29	10 "	55	13.1	"
R. Itchen	July 5	9 "	99	12.6	"
	" 6	9 "	106	12.6	Some sunshine
R. Itchen	" 27	9 "	93	12.8	Dull
	" 28	9 "	113	13.4	"
R. Lark	Aug. 4	9 "	58	16.25	Sunshine
	" 5	9 "	53	16.1	"
R. Itchen	" 23	9 "	105.5	12.3	"
	" 24	9 "	107	11.0	"
R. Lark	Sept. 7	10 "	38	14.7	Dull
	" 8	10 "	55	14.0	Bright
R. Lark	" 22	8 "	26	14.9	Dull
	" 23	8 "	35	11.95	Bright
R. Itchen	" 30	7 "	76	6.2	"
	Oct. 1	7 "	80	9.0	Dull
R. Lark	" 12	10 "	36	10.5	"
	" 13	10 "	31	9.95	"
R. Itchen	Nov. 10	8 "	82	3.9	Bright sun
	" 11	8 "	81	4.1	"
R. Itchen	Dec. 14	10 "	88	6.0	Rain
	" 15	10 "	89	6.0	Dull. Fine

It will be seen that these day-to-day differences in oxygen saturation do not follow the water temperature, but are the result of the variation in actinic conditions. The variation in this respect, however, is small in comparison with the variation due to seasonal changes in plant life. Whereas the difference

between the highest and lowest maximum oxygen saturation throughout the year is 111 %, due to the combined effects of the variation in plant life and of the variation in actinic conditions, the greatest difference from day to day, due to actinic conditions only, is 26 % over this small collection of data.

The results given in Tables XV, XVI and XVII were obtained during the period of pollution of the River Lark by the effluents from the beet-sugar factory at Bury St Edmunds. In spite of this pollution the curves for October 12-13 and November 2-3 still indicate a small diurnal variation showing that there is still some photosynthetic activity.

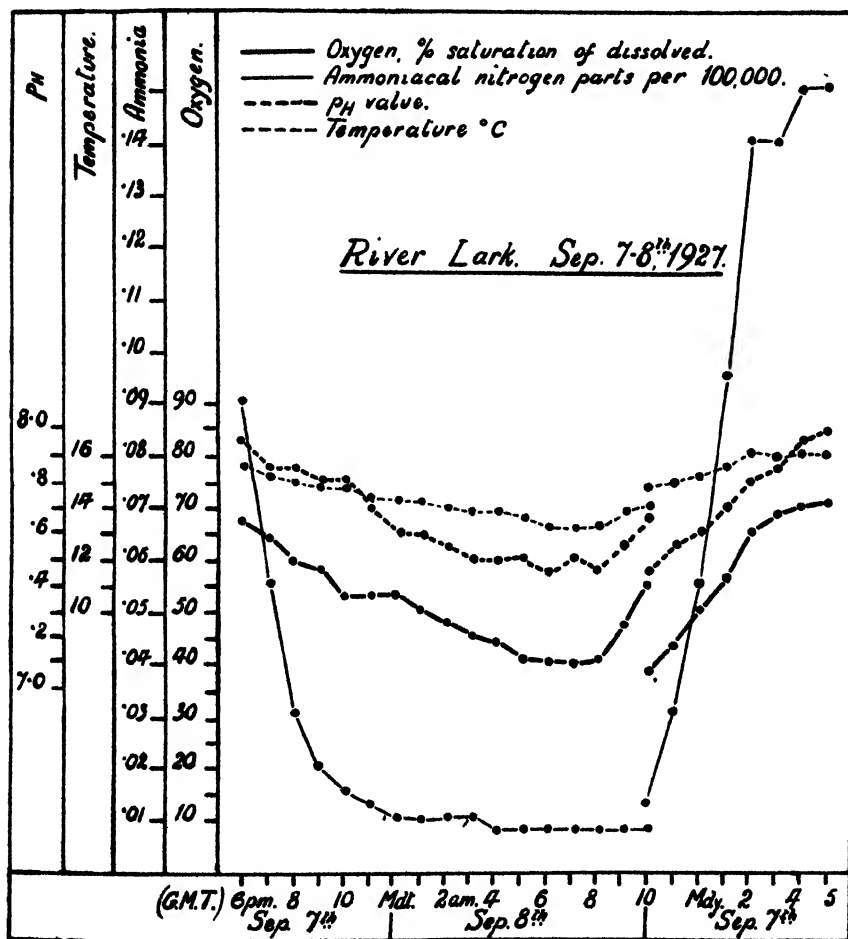


Fig. 11.

*River Itchen. August-December.* On the River Itchen the oxygen saturation curves (Figs. 15-18) show a progressive lowering of the maximum value and a progressive increase in the minimum value; the range of variation decreases regularly from 48 % of saturation in August to 6 % in December.

During the winter the decrease in the number of water organisms and the retardation of the respiration of the remaining organisms due to the lower temperature result in a decreased oxygen absorption and a consequent increase in the minimum value of oxygen saturation.

Table XIII. *River Lark. Series VI. September 7-8, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	pH	Remarks
		Parts per 100,000	% saturation			
10 a.m.	14.7	0.393	38	0.01-0.015	7.4-7.5	Dull
11 "	14.9	0.442	43	0.03	7.5-7.6	Sun
Midday	15.2	0.514	50	0.05-0.06	7.6	Sun and cloud
1 p.m.	15.55	0.572	56	0.09-0.1	7.7	"
2 "	16.0	0.653	65	0.14	7.8	Bright
3 "	15.9	0.688	68	0.14	7.8-7.9	Cloudy
4 "	16.0	0.710	70	0.15	7.9-8.0	Sunny
5 "	16.0	0.719	71	0.15	8.0	"
6 "	15.6	0.688	67	0.09	7.9-8.0	Sun going down
7 "	15.3	0.653	64	0.05-0.06	7.8-7.9	Dark
8 "	15.0	0.617	60	0.03	7.8-7.9	Moonlight
9 "	14.8	0.596	58	0.02	7.8	"
10 "	14.7	0.555	53	0.015	7.8	"
11 "	14.45	0.555	53	0.01-0.015	7.7	"
Midnight	14.35	0.555	53	0.01	7.6	"
1 a.m.	14.2	0.524	50	0.01	7.6	Moonset
2 "	13.95	0.504	48	0.01	7.5-7.6	"
3 "	13.8	0.473	45	0.01	7.5	"
4 "	13.8	0.458	44	<0.01	7.5	Sky just lightening
5 "	13.5	0.439	41	<0.01	7.5	Sunrise
6 "	13.3	0.429	40	<0.01	7.4-7.5	Misty bright
7 "	13.1	0.421	39	<0.01	7.5	Bright
8 "	13.2	0.432	40	<0.01	7.4-7.5	"
9 "	13.7	0.493	47	<0.01	7.5-7.6	"
10 "	14.0	0.575	55	<0.01	7.6-7.7	"

Sunrise 5.25 a.m.

Sunset 6.30 p.m.

It would appear that the progressive lowering of the maximum value of saturation is due to the decrease in temperature, since the oxygen expressed in parts per 100,000 is practically constant from June to December—November and December showing slight differences which are accounted for by prevailing actinic conditions, while the percentage saturation shows considerable variation. These results give some information on the relative effects upon the oxygen saturation of river waters of such factors as photosynthesis, respiration, temperature, absorption from air, which will be discussed further in Part IV when the completed data for a year will be presented.

#### *Ammoniacal nitrogen curves.*

*River Lark.* On the River Lark, the values of ammoniacal nitrogen for September. October and November vary roughly in a sense opposite to those of dissolved oxygen with the exception of those obtained on September 7-8; this set of observations, however, was made under abnormal conditions, since extensive weed cutting had taken place for one mile above the point of sampling, and large masses of dead and decaying weeds had been left in this stretch of the river.

Table XIV. *River Lark. Series VII. September 22-23, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	pH	Remarks
		Parts per 100,000	% saturation			
8 a.m.	14.9	0.269	26	0.05	7.5	Dull. Rain.
9 "	15.0	0.296	29	0.05-0.06	7.5-7.6	" " Windy
10 "	15.0	0.316	30.5	0.05-0.06	7.5-7.6	" " "
11 "	15.1	0.385	38	0.05-0.06	7.5-7.6	" Fine
Midday	15.2	0.464	45	0.05-0.06	7.6	" Rain
1 p.m.	15.4	0.524	51	—	> 7.6	" "
2 "	15.3	0.573	55	0.05-0.06	7.6-7.7	" "
3 "	15.2	0.632	61	0.04	7.7	" "
4 "	15.05	0.652	63	0.03	> 7.7	" " Little sun
5 "	14.95	0.709	70	0.015	7.7-7.8	" Fine
6 "	14.7	0.700	68	0.01-0.015	7.8	" "
7 "	14.45	0.681	66	0.01	7.7-7.8	Fine. Dark
8 "	14.05	0.611	58	0.01	7.7	" " No moon
9 "	14.0	0.566	54	0.01	7.6-7.7	" "
10 "	13.45	0.504	48	0.01	7.6	" "
11 "	13.5	0.484	46	0.01	7.5-7.6	" "
Midnight	13.4	0.443	42	0.01	7.5-7.6	" "
1 a.m.	13.35	0.429	40	0.01	7.5-7.6	" "
2 "	13.1	0.425	40	0.01	7.5-7.6	" "
3 "	12.9	0.405	38	0.01	7.5-7.6	" "
4 "	12.7	0.395	37	0.01	7.5-7.6	" "
5 "	12.5	0.361	33	0.02	7.5-7.6	Dawn. Fine
6 "	12.3	0.362	33	0.03	7.5-7.6	Sunrise. Clear
7 "	12.2	0.375	34	0.03	7.5	Clear. Sunny
8 "	11.95	0.385	35	0.04-0.05	7.5	" "
9 "	12.0	0.449	41	0.05	7.5-7.6	" "

Sunrise 5.50 a.m.

Sunset 6.0 p.m.

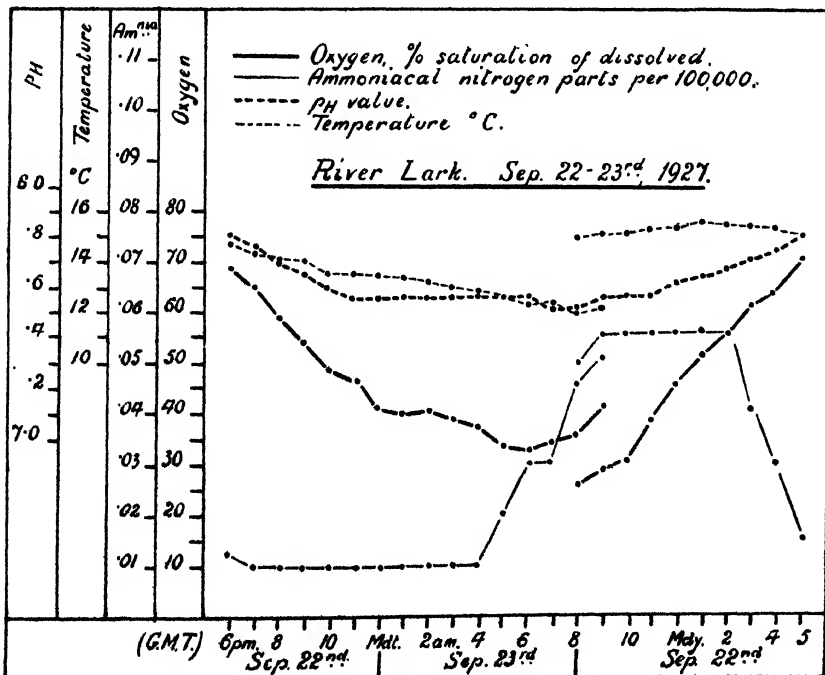


Fig. 12.

Table XV. *River Lark, Series VIII. October 12-13, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	p <sub>H</sub>	Remarks
		Parts per 100,000	% saturation			
10 a.m.	10.5	0.405	36	0.1	7.5	Dull. Fine. River clear
11 "	10.5	0.415	37	0.1	7.5-7.6	Dull. Drizzle
Midday	10.5	0.435	38.5	0.1	<7.6	" Fine
1 p.m.	10.6	0.461	41	0.1	7.7	" "
2 "	10.6	0.506	45	0.08	7.7-7.8	
3 "	10.6	0.555	49	0.08	7.7-7.8	
4 "	10.6	0.553	49	0.04-0.05	7.8	
5 "	10.6	0.523	46	0.03	7.7	Dull. Almost dark. Fine
6 "	10.6	0.405	36	0.015	7.7	Dark. Fine
7 "	10.6	0.456	40	0.01	7.6	" "
8 "	10.6	0.499	44	0.01	7.6	
9 "	10.6	0.497	44	<0.01	7.6	
10 "	10.6	0.496	44	<0.01	7.5	
11 "	10.6	0.475	44	<0.01	7.5	Drizzle
Midnight	10.5	0.464	41	<0.01	7.5	"
1 a.m.	10.5	0.443	39	<0.01	7.5	"
2 "	10.5	0.446	39	<0.01	<7.5	"
3 "	10.4	0.435	38	<0.01	<7.5	"
4 "	10.4	0.425	37	<0.01	<7.5	"
5 "	10.3	0.385	34	0.01	7.4-7.5	Daylight. Dull. Hazy
6 "	10.2	0.336	30	0.04	7.4-7.5	
7 "	10.15	0.309	27	0.09	7.4-7.5	Dull
8 "	10.1	0.324	28	0.11	7.5	"
9 "	9.9	0.324	28	0.1	7.5	"
10 "	9.95	0.359	31	0.1	7.5	"

Sunrise 6.20 a.m.

Sunset 5.10 p.m.

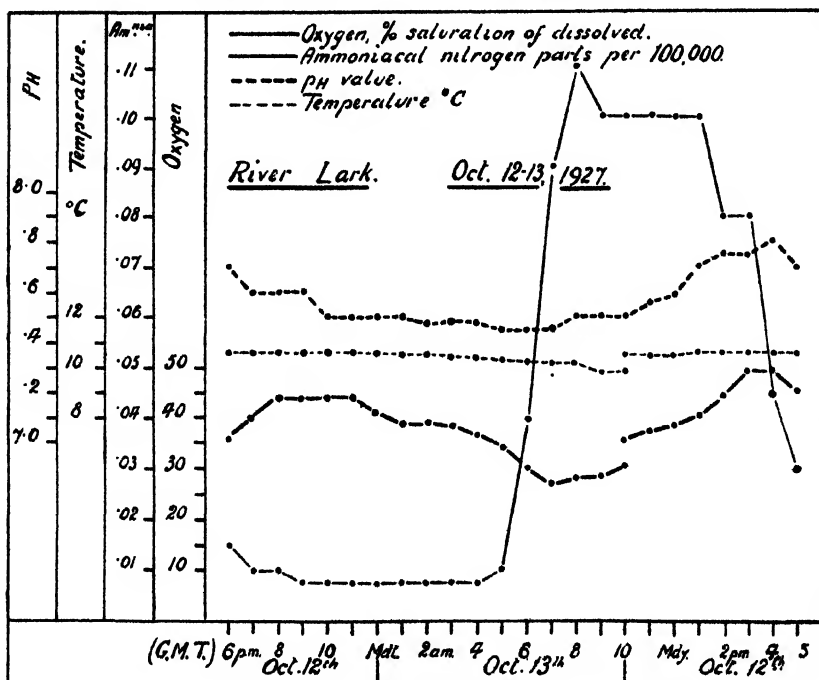


Fig. 13.

Table XVI. *River Lark. Series IX. November 2, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen.		pH	Remarks
		Parts per 100,000	% saturation	Parts per 100,000			
10.30 a.m.	13.1	0.295	28	0.04		7.5-7.6	During pollution by the effluents from the beet-sugar fac- tory at Bury St Edmunds
11.45 "	13.2	0.338	31.5	0.03		7.5-7.6	
12.55 p.m.	13.35	0.359	34	0.02		7.6	
2.20 "	13.6	0.352	33	0.01		7.6	
3.30 "	13.7	0.327	31	0.01-0.015		7.6	
4.45 "	13.9	0.295	28	0.01-0.015		7.5-7.6	
6.40 "	13.8	0.264	25	0.01-0.015		7.5-7.6	
9.30 a.m. (Nov. 3rd)	13.1	0.137	12	0.03		7.5-7.6	

Sunrise 6.55 a.m.

Sunset 4.32 p.m.

Table XVII. *River Lark. Series X. November 17-18, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen.		pH	Remarks
		Parts per 100,000	% saturation	Parts per 100,000			
1 p.m.	8.2	0.083	7	0.16		7.5	Fine. Clear " " Dusk Dark
2 "	8.1	0.124	10	0.08		7.5-7.6	
3 "	7.9	0.114	9.5	0.06		7.6	
4 "	7.7	0.072	6	0.05		7.5-7.6	
5 "	7.6	0.083	7	0.045		7.5-7.6	Dusk Dark
6 "	7.6	0.119	10	0.04		7.5-7.6	
7 "	7.5	0.089	7.5	0.03		7.5-7.6	
8 "	7.45	0.092	8	0.025-0.03		7.5-7.6	
9 "	7.4	0.093	8	0.025		7.5-7.6	Dusk Dark
10 "	7.3	0.094	8	0.025		7.5-7.6	
11 "	7.2	0.074	6	0.03		7.5-7.6	
Midnight	—	—	—	—		—	
1 a.m.	7.3	0.042	3.5	0.08		7.5-7.6	Dusk Dark
2 "	7.3	0.030	2.5	0.13		7.5-7.6	
3 "	7.3	0.035	3	0.14		7.5-7.6	
4 "	7.4	0.031	2.5	0.16		7.5	
5 "	7.4	0.031	2.5	0.16		7.4-7.5	Dusk Dark
6 "	7.5	0.031	2.5	0.16		7.4-7.5	
7 "	7.5	0.027	2	0.16		7.4-7.5	
8 "	7.5	0.022	2	0.16		7.5	
9 "	7.5	0.031	2.5	0.16		7.5	Dusk Dark
10 "	7.5	0.034	3	0.16		7.5	
11 "	7.6	0.021	2	0.16		7.5	
Midday	7.9	0.019	2	0.16		7.5	
1 p.m.	8.1	0.021	2	0.08		7.5	

Sunrise 6.30 a.m.

Sunset 5.0 p.m.

This experiment was made after the River Lark had been seriously polluted for a period of 6 weeks by the effluents from the beet-sugar factory.

In general, the commencement of the period of maximum of ammoniacal nitrogen follows just after the commencement of the period of minimum of oxygenation, and *vice versa*.

In the bacterial nitrification of ammonia, the effects of such factors as concentration of dissolved oxygen, mechanical aeration, presence of suspended particles and of dissolved salts, temperature, etc., are considerable, and the data obtained during these experiments are insufficient to give a complete explanation of these abnormal ammoniacal nitrogen figures.

There can be little doubt, however, that the accumulation of organic matter at the point of sampling during the pollution of the river bed has led to this abnormal production of ammonia and amino-compounds; it was noted during the estimations of ammoniacal nitrogen that the greenish tinge indicative of amino-compounds was usually obtained on addition of Nessler's reagent.

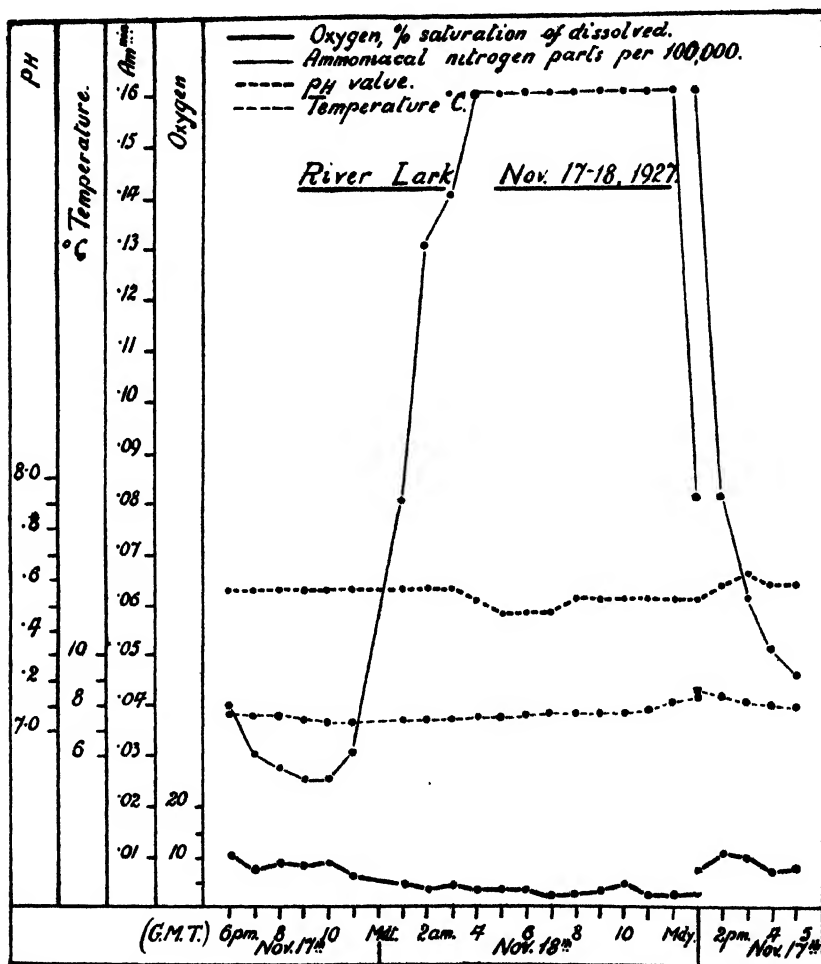


Table XVIII. *River Itchen. Series V. August 23-24, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH	Remarks
		Parts per 100,000	% saturation			
9 a.m.	12.3	1.149	105.5	0.005	7.9	Fine. Sunny. Occasional cloud
10 "	12.6	1.192	110	0.005	8.0	" "
11 "	13.2	1.200	112	0.005	8.1	" "
Midday	14.4	1.213	117	0.005	8.2	Fine. Cloudy. Occasional sun
1 p.m.	14.4	1.190	114	0.005	8.2	Raining slightly
2 "	14.3	1.129	108	0.005	8.2	Dull. Fine. Thundery
3 "	14.9	1.216	118	0.005	8.2	Fine. Occasional sun
4 "	14.8	1.186	115	0.005	8.2	Raining slightly
5 "	14.2	1.150	110	0.005	8.0	" "
6 "	13.9	1.099	105	0.005	8.0	Sunshine
7 "	13.1	0.971	91	0.005	7.8	" "
8 "	12.9	0.880	82	0.005	7.7	Cloudy, almost dark
9 "	12.2	0.823	75.5	0.005	7.6	Dark, some cloud
10 "	11.9	0.838	76	0.005	7.6	Clear and stormy
11 "	11.1	0.822	73	0.005	<7.6	Ground mist
Midnight	10.9	0.809	72	0.005	7.5	" "
1 a.m.	10.4	0.803	70	0.005	7.5	" "
2 "	10.0	0.801	70	0.005	7.4-7.5	" "
3 "	9.9	0.827	72	0.005	7.4-7.5	" Moon shining
4 "	9.8	0.817	71	0.005	7.4	" Dawn
5 "	9.6	0.825	71	0.005	7.4-7.5	" Daylight
6 "	9.3	0.873	75	0.005	7.5	Sun shining through mist
7 "	9.3	0.971	86	0.005	7.5-7.6	Sunshine. Mist clearing
8 "	10.0	1.093	95	0.005	7.8	" "
9 "	11.0	1.195	107	0.005	7.9	" Some cloud

Sunrise August 23, 4.57 a.m.

Sunset " 23, 7.7 p.m.

Sunrise " 24, 4.59 a.m.

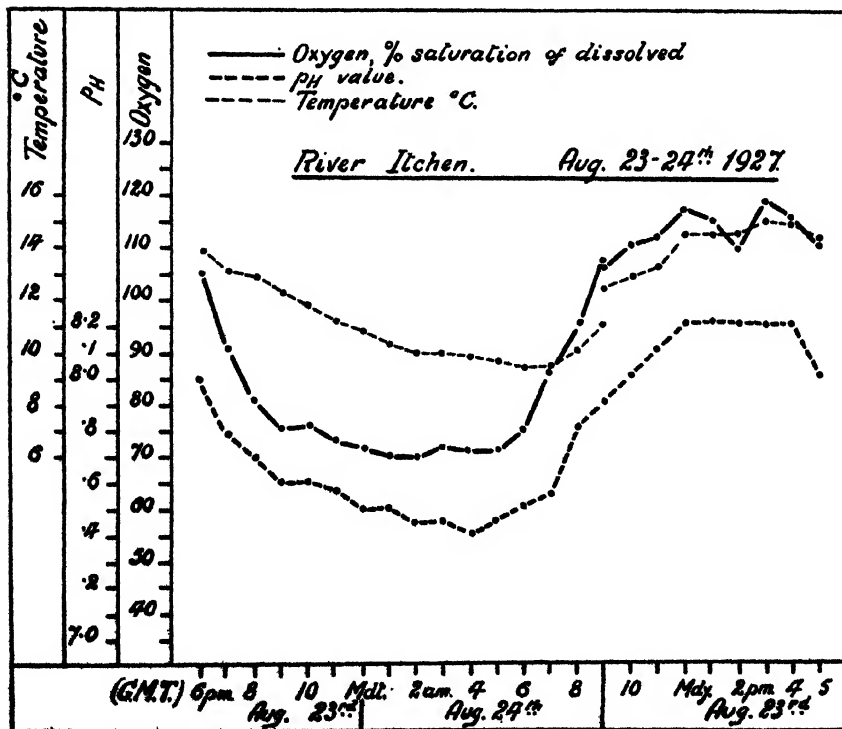


Fig. 15.



Table XIX. *River Itchen. Series VI. September 30–October 1, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH	Remarks
		Parts per 100,000	% saturation			
7 a.m.	6.2	0.956	76	0.005	7.5	River high but clear. Sunshine. Ground frost
8 "	6.1	1.057	84	0.005	7.5-7.6	Sunshine
9 "	6.8	1.124	91	0.005	7.6	"
10 "	7.8	1.184	98	0.005	7.6	Some cloud. Weed-cutting
11 "	8.5	1.188	100	0.005	7.7	Water turbid due to weed- cutting
Midday	9.3	1.202	104	0.005	7.8	Sunshine. Weed-cutting stop- ped. Water clear
1 p.m.	10.0	1.181	103	0.005	7.9	Sunshine
2 "	10.5	1.181	104	0.005	7.9	"
3 "	11.0	1.186	106	0.005	8.0	"
4 "	10.8	1.126	99	0.005	7.9	Dull
5 "	10.3	1.080	95	0.005	7.9-7.8	"
6 "	10.1	1.013	90	0.005	7.8	" Sunset
7 "	9.8	0.954	83	0.005	>7.7	Cloudy. Dark
8 "	9.7	0.908	78	0.005	7.7	"
9 "	9.6	0.904	78	0.005	<7.7	"
10 "	9.5	0.893	77	0.005	>7.6	"
11 "	9.4	0.872	75	0.005	7.6	"
Midnight	9.4	0.872	75	0.005	<7.6	"
1 a.m.	9.3	0.903	78	0.005	<7.6	Sky clear
2 "	9.1	0.886	76	0.005	<7.6	"
3 "	9.0	0.873	76	0.005	<7.6	Some cloud
4 "	9.0	0.897	77	0.005	<7.6	Storm at 4.30
5 "	9.0	0.899	78	0.005	<7.6	Dull. Sky just lightening
6 "	9.0	0.886	76	0.005	<7.6	Daylight. Raining slightly
7 "	9.0	0.932	80	0.005	7.5	Raining

Sunrise September 30, 5.58 a.m.

Sunset " 30, 5.41 p.m.

Sunrise October 1, 6.0 a.m.

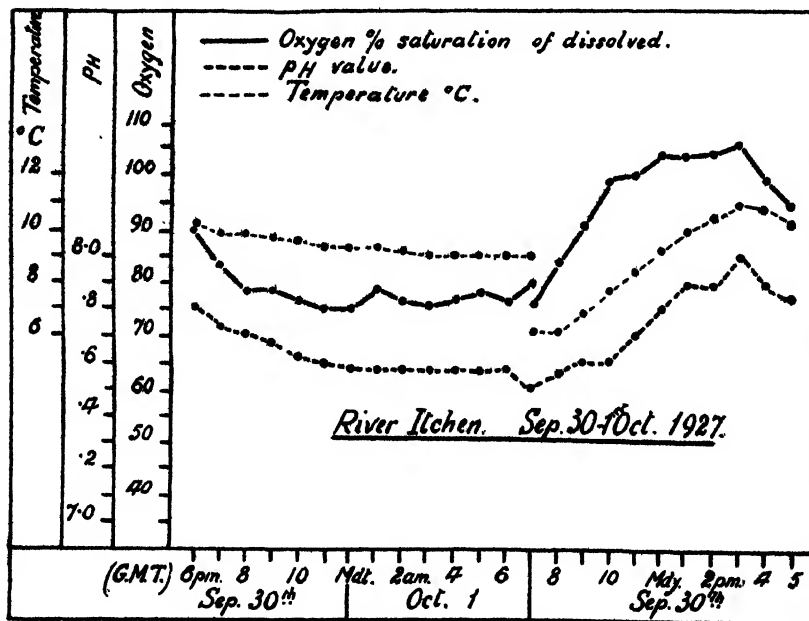


Fig. 16.

Table XX. *River Itchen. Series VII. November 10-11, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH	Remarks
		Parts per 100,000	% saturation			
8 a.m.	3.9	1.080	82	0.005	7.8	Bright sun. Frost
9 "	4.0	1.148	89	0.005	7.8	Sunshine
10 "	4.6	1.194	92	0.005	7.8-7.9	"
11 "	5.4	1.250	98	0.005	7.9	Cloudy. Occasional sun. Cold wind
Midday	6.0	1.260	100	0.005	7.9	Sunshine. Cold wind
1 p.m.	6.2	1.250	100	0.005	7.9	Sunshine. Some cloud. Cold wind
2 "	6.2	1.238	100	0.005	7.9	Sunshine. Some cloud. Cold wind
3 "	6.2	1.217	97	0.005	7.9	Sunshine
4 "	6.0	1.179	94	0.005	7.9	" Sun setting
5 "	5.7	1.105	87	0.005	7.9-7.8	Dusk. Fine
6 "	5.5	1.077	84	0.005	7.9-7.8	Dark. Cloudy
7 "	5.5	1.070	84	0.005	7.8	" "
8 "	5.4	1.066	83	0.005	7.8	" "
9 "	5.3	1.047	82	0.005	7.8	" "
10 "	5.1	1.056	82	0.005	7.8	Moonlight. Clear
11 "	4.9	1.051	81	0.005	7.8	" "
Midnight	4.7	1.056	81	0.005	7.8-7.7	" "
1 a.m.	4.5	1.038	80	0.005	7.8-7.7	" "
2 "	4.5	1.052	81	0.005	7.8-7.7	" "
3 "	4.5	1.054	81	0.005	7.8-7.7	" "
4 "	4.4	1.053	81	0.005	7.8-7.7	" "
5 "	4.3	1.053	81	0.005	7.8-7.7	" "
6 "	4.2	1.056	80	0.005	7.8-7.7	Moonlight. Sky just lightening
7 "	4.2	1.077	82	0.005	7.8-7.7	Daylight. Clear
8 "	4.1	1.072	81	0.005	7.8-7.7	Sunshine

Sunrise November 10, 7.8 a.m.

Sunset " 10, 4.19 p.m.

Sunrise " 11, 7.10 a.m.

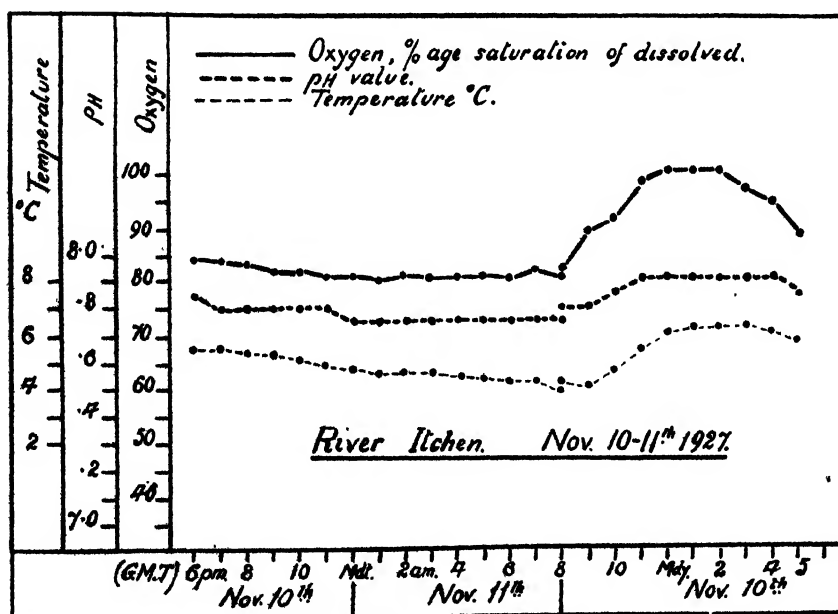


Fig. 17.

Table XXI. *River Itchen. Series VIII. December 14-15, 1927.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	$p_H$	Remarks
		Parts per 100,000	% saturation			
10 a.m.	6.0	1.104	88	0.015	7.8	Rain. Water turbid
11 "	6.0	1.101	87	0.015-0.01	7.8	
Midday	6.0	1.101	87	0.015-0.01	7.8	Dull. "Fine." Water turbid
1 p.m.	6.0	1.109	88	0.015-0.01	7.8	
2 "	6.0	1.105	88	0.015-0.01	7.8	Slight rain. "Water" turbid
3 "	6.1	1.089	86	0.015-0.01	7.8	" " "
4 "	6.1	1.085	86	0.01-0.005	7.8	" " "
5 "	6.2	1.075	86	0.01-0.005	7.8	" " " Dark
6 "	6.3	1.064	85	0.01-0.005	7.8	" " " "
7 "	6.4	1.070	86	0.01-0.005	7.8	Dull. "Fine." Dark
8 "	6.4	1.059	85	0.01-0.005	7.8	" " " "
9 "	6.4	1.055	84	0.01-0.005	7.8	Rain. Dark "
10 "	6.4	1.055	84	0.01-0.005	7.8	Dull. Fine. Dark
11 "	6.3	1.055	84	0.01-0.005	7.8	" " " "
Midnight	6.2	1.055	84	0.01-0.005	7.8	" " " "
1 a.m.	6.1	1.043	83	0.01-0.005	7.8	" " " "
2 "	6.2	1.055	84	0.01-0.005	7.8	" " " "
3 "	6.3	1.055	84	0.01-0.005	7.8	" " " "
4 "	6.3	1.055	84	0.01-0.005	7.8	" " " "
5 "	6.2	1.055	84	0.01-0.005	7.8	Drizzle. Dark "
6 "	6.1	1.059	84	0.01-0.005	7.8	" " " "
7 "	6.0	1.071	85	0.01-0.005	7.8	Drizzle. Sky just lightening
8 "	6.0	1.075	85	0.01-0.005	7.8	Dull. Fine. Daylight
9 "	6.0	1.095	87	0.01-0.005	> 7.8	" " Water clearer
10 "	6.0	1.116	89	0.01-0.005	> 7.8	" " " "

Sunrise December 14, 7.58 a.m.

Sunset " 14, 3.51 p.m.

Sunrise " 15, 7.59 a.m.

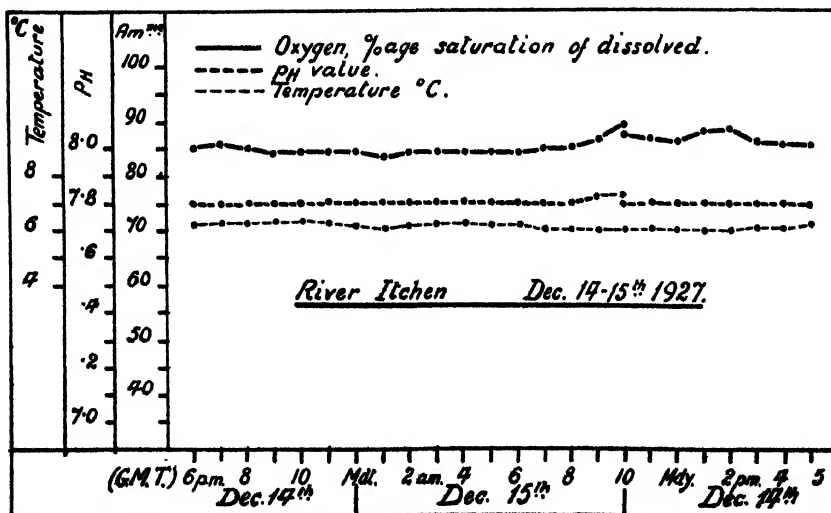


Fig. 18.

parts per 100,000, although occasional higher values 0.01 and 0.015 were obtained, and were probably due to contamination of the river by soil drainage during rainy periods.

*p<sub>H</sub> curves.* The *p<sub>H</sub>* curves are again similar in structure and in general follow the oxygen curves, the range in the winter being less than that in the spring and summer. This decrease in the range of *p<sub>H</sub>* variation is accounted for by the decrease in photosynthesis and the lessened respiration mentioned in the discussion of the oxygen curves.

*Water temperature curves.* The temperature curves show the expected diurnal similarity but over different ranges according to the month of the year and the prevailing weather conditions.

In conclusion the authors wish again to thank Sir R. Robertson, the Government Chemist, and the Ministry of Agriculture and Fisheries, for permission to publish these results.

#### REFERENCE.

Butcher, Pentelow and Woodley (1927). *Biochem. J.* **21**, 945, 1423.

# CXXIX. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA.

## PART III. ON THE SO-CALLED ALIMENTARY HYPERCHOLESTEROLAEMIA.

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*(Received June 20th, 1928.)*

As far as the exogenous portion of the blood cholesterol is concerned, the work of the past 20 years has made it perfectly clear that in animals most of the cholesterol which is absorbed from the food reaches the blood stream *via* the lymphatic channels and the thoracic duct [Müller, 1915, 1916]; some, however, is absorbed directly from the intestines. It would appear probable that, if the cholesterol content of the blood is increased by this process, the increase would be mainly if not entirely in the plasma. The red blood corpuscles contain their cholesterol almost wholly in the free condition and probably as an integral part of the cell structure. Experiments which have been made on pure isolated red blood cells indicate that their cholesterol content is remarkably constant in a given animal in health or disease. The experimental data are not, however, numerous, and the conclusion has been denied by observers who have determined the cholesterol content of these cells indirectly by estimating the cholesterol, free and ester, of the whole blood and also of the plasma, using the haematocrit to ascertain the proportion of red cells in the whole blood. Indirect methods of this type lead to results which are open to serious doubt owing to inherent errors in the estimation of the true volume of the red cells by the haematocrit or other methods. On the other hand, the isolation of pure red blood corpuscles perfectly free from plasma constituents is a tedious and difficult operation.

In the human subject the data in the literature on the question of alimentary hypercholesterolaemia are very conflicting. Widal, Weill and Laudat [1912] stated that in healthy subjects, who had fasted for 15 hours, a meal consisting of meat, potatoes, bread and 50 g. of butter caused a marked increase in the cholesterol of the blood. Grigaut [1913], commenting on these results, pointed out that the actual total increase of cholesterol in the whole of the blood was many times greater than the amount of cholesterol ingested. Hence he suggested, without other evidence, that a certain quantity of cholesterol is formed at the expense of the ingested fat. He also pointed out that, in contrast

to pathological hypercholesterolaemia, alimentary hypercholesterolaemia is transitory and there is a return to normal soon after the cessation of the special feeding. Rouzaud and Cabanis [1913] fed 11 healthy individuals with a meal containing soup, bread, meat, green vegetables, two eggs and wine, and found no difference in the cholesterol of the blood taken before and 4 to 5 hours after this meal. There was one case which showed an increase of 5 %. Luden [1917], in experiments on herself, found that, whereas a light meal of bread and butter and tea had no effect on the blood cholesterol, a somewhat heavier meal containing bread, butter, tea, one egg and half a cantaloup produced an increase of blood cholesterol of about 25–30 %. Here again, if we calculate from the figures given, the increase in blood cholesterol is greater than could be accounted for by the cholesterol ingested. She also states that on prolonged feeding with a vegetable diet, blood cholesterol is decreased, and with prolonged feeding with meat, blood cholesterol is increased. Denis [1917] fed three men with a heavy fat meal and found that no increase of blood cholesterol followed.

In all these experiments, however, the total cholesterol only was estimated, as though free cholesterol and ester cholesterol had an identical significance, an assumption by no means proven. Further, the estimations were made by colorimetric methods against which serious objections have been raised.

Bürger and Habs [1927] reported the results of feeding "starving" patients, suffering from various complaints, though presumed to be metabolically sound, on a breakfast consisting of 100 g. of olive oil in which had been dissolved 5 g. of cholesterol or its equivalent of cholesterol esters. They found in the serum a marked hypercholesterolaemia, reaching a maximum about 4 hours after the meal. This more or less rapidly decreased again during 24 hours to normal. The ratio of free to ester cholesterol during these experiments was stated to remain constant, though the figures in some of the cases scarcely bear out this conclusion. Although they used Windaus's method of estimating the extracted sterols, a consideration of their methods and the rather low results they obtained suggests to us that the mode of extraction used was probably inefficient. There is however the further difficulty of drawing conclusions, which are applicable to normal states, from experiments in which a meal of such an unphysiological nature is fed. In these experiments the excess of fat has clearly increased the rate of absorption and quantity of the ingested sterols absorbed.

It seemed to us desirable to investigate the subject of alimentary cholesterol-aemia more closely by means of the digitonin method of estimation, which permits of separate estimation of the free cholesterol and that in form of esters. The details of the methods used have been fully discussed in Part I of this series [1927, 1].

#### EXPERIMENTAL.

In experiments dealing with the effect of a single meal, it is not surprising that the results should be somewhat inconclusive, for simple calculation will

show that a meal containing, say, 0.2 g. of cholesterol, even making the improbable assumption that the latter is completely absorbed, could only raise the average content of the plasma of a man weighing 70 kg. by less than 0.007 %, which is scarcely beyond the limits of the error of experiment.

However, our experiments show that it is possible to vary the cholesterol level of the plasma in man, just as in animals, by sufficiently prolonged feeding with diets of high or low sterol content, the blood specimens for analysis being *always* taken when the subject is in a *fasting condition* (e.g. before breakfast) so as to eliminate, as far as possible, any disturbing factors, such as digestion or muscular work, which might affect the general composition of the plasma. The following experiment illustrates this.

The subject of the experiment was a man, aged 40, weighing about 8 stone, who had been in hospital for some time with a mild colitis and was convalescing. He had been fed for 24 days on a light hospital diet (A) consisting of bread, butter, milk and puddings, of approximately 1900-2000 calories per day; the cholesterol content of the diet was calculated to be approximately 0.32 g. per day. During the following 7 days he was put on a diet (B) which was almost sterol-free and consisted largely of white of egg, potato and bread, supplemented by porridge and treacle, and an apple or orange. For drink he had bovril, tea and beer. The daily calorie value was about 1500 and the sterol content approximately 0.06 g. Care was taken that this diet was cooked in as appetising a manner as possible, with the result that the patient ate it readily and made no complaint. During this period he lost 2-3 lb. in weight. He was then, for a further period of 7 days, given an ordinary full hospital diet (C), enriched in cholesterol by the addition of about 200 g. of calves' brain daily. The calorie value was about 2500 and the sterol content about 4.0 g. per day. His weight remained unchanged. For the following period of 11 days he was given a full diet (D) consisting of fish, two eggs, bread, butter and potatoes and fresh milk; the calorie value was about 2200 and the sterol content about 0.94 g. daily. During the whole of the experiment the patient remained in bed and the samples of blood for analysis, of about 20-25 cc., were taken at the end of each experimental period at about 11.30 a.m., the patient having only had a cup of tea at 6.0 a.m. As far as possible physiological requirements were carefully considered in constructing the diets, but of necessity the low cholesterol diet was deficient in fat and the brain diet had an excess of ether-extractable matter.

The results are given in Table I.

Table I.

Diet	Approximate sterol intake per day (g.)	Duration of diet (days)	% of cholesterol in plasma taken at end of feeding period and "starving"			% of total cholesterol as ester
			free	ester	total	
A	0.32	24	0.0500	0.0653	0.1153	56.6
B	0.06	7	0.0400	0.0319	0.0719	44.3
C	4.0	7	0.0413	0.0808	0.1221	66.1
D	0.94	11	0.0504	0.1044	0.1548	67.5

It will be noticed that the free cholesterol of the plasma maintains approximately the same level even after such totally different diets as B and C, but on the other hand the amount of ester cholesterol in the plasma appears definitely to follow the cholesterol content of the food. It will be noticed, however, that the increase of ester cholesterol with the brain diet is not so much as with the more normal diet D. Possibly the explanation of this is that brain is not so easily absorbed as other forms of food, or that the brain cholesterol is more readily reduced in the intestine to coprosterol and so not absorbed. In this connection we may recall a similar effect obtained by Dorée and Gardner [1908] in experiments on dogs. Also if Wieland and Sorge's "choleic acid principle" [1916] be valid, the amount of cholesterol absorbed from food might perhaps be limited by the quantity of deoxycholic acid derivatives in the bile passing into the intestines.

*Experiments on the effect of a single full meal.*

The next series of experiments was designed to investigate the effect of a single full meal on a fasting individual.

The first specimen of blood (20–30 cc.), from a vein in the arm, was taken about 1 hour before the meal was given, the subject having fasted since the previous evening (referred to in the table as "fasting"); the later specimens of about the same volume were taken during the course of digestion. The meals were substantial and, except those of very low sterol content, of more or less normal dinner character. They contained varying amounts of sterol, some being nearly free and others enriched, as regards sterol, by adding such constituents as eggs or calves' brain.

The results are set out in Table II.

DISCUSSION.

The results of these single meal experiments are conflicting and we cannot yet account for all the phenomena presented. At first sight it might be thought that some of the variations were due to errors of experiment, since, for obvious reasons, the analyses were not done in duplicate. The analyses, however, were made on quite adequate weights of plasma—10 to 14 g.—and our long experience of the method rules out errors of manipulation, beyond such slight errors as are inevitable in all gravimetric procedure.

It might well be, however, that the samples of blood drawn from an arm vein did not accurately represent the average composition of the whole blood, more especially during active digestion. Such error would, however, we think, tend to smooth out variations rather than accentuate them.

The first three experiments tabulated were done with meals of very low sterol content, and consequently of sub-normal fat content. The results are concordant in showing a small but definite lowering of the total cholesterol content of the plasma.

In Exp. 1, on a quite normal individual, the lowering is practically confined



Table II. *Normal healthy subjects.*

Exp. no.	Date of experiment	Subject	Composition of meal	Approx. sterol intake (g.)	Time of taking plasma after meal	Cholesterol content of plasma (g. %)			Remarks	
						free	ester	total		
1	17. iii. 1927	J. A. G.	Potatoes 450 g., whites of 5 eggs, 14 g. butter, slice of brown bread, 1 pint beer	— 0.04	Fasting 3½ hours	0.0778 0.0753	0.1302 0.1024	0.2080 0.1782	Clear yellow, no haemolysis Slight opalescence, no haemolysis	
2	21. xii. 1927	X.	—	—	Fasting 3½ hours	0.1027 0.0768	0.1906 0.1744	0.2935 0.2512	Clear brown, no haemolysis Clear brown, no haemolysis	Non-sterol unsap. matter 0.2466 % 0.1670 %
3	24. xii. 1927	W.	—	—	Fasting 3½ hours	—	—	0.2295 0.1858	Plasma clear, very slight haemolysis Plasma clear, very slight haemolysis	Non-sterol unsap. matter 0.3201 % 0.3767 %
4	19. v. 1925	J. A. G.	Turtle soup, saddle of mutton, peas, potatoes, bread, butter, cheddar cheese, radishes, ½ pint Chamberlain and coffee	— 0.25-0.35	Fasting 2½ hours	0.0514 0.0404	0.1388 0.1210	0.1900 0.1614	Plasma yellow, clear, no haemolysis Plasma darker and greenish, milky, no haemolysis	
5	19. v. 1925	H. G.	—	— 0.25-0.35	Fasting 2½ hours	0.0418 0.0375	0.1142 0.0809	0.1560 0.1184	Plasma yellow, clear, no haemolysis Plasma milky, greenish colour, no haemolysis	
6	20. iv. 1927	S.	Double helping lean meat, 1 small potato, green vegetables, 80 g. cheese, 1 bottle beer	— 0.25-0.3	Fasting 2½ hours	0.0514 0.0547	0.0797 0.0899	0.1311 0.1446	Plasma clear, slight haemolysis Plasma opalescent, slight haemolysis	20 grains of Fei bovinum 1 hour before meal
7	15. v. 1928	J. A. G.	Turtle soup ½ pint, 252 g. steak (182 g. after cooking), 1 tomato, peas, fried potatoes, fresh raspberries, 15 g. cream, toast, 1½ g. butter, 1 pint claret	— 0.2-0.3	Fasting 2½ hours 4½ hours	0.0613 0.1135 0.1715	0.1388 0.0974 0.0488	0.2001 0.2109 0.2203	Clear yellow, no haemolysis Clear, darker, greenish, fluorescence Faintly opalescent, no haemolysis	Non-sterol unsap. matter 0.2558 % 0.4243 % 0.4943 %
8	15. v. 1928	G. E.	Ditto, but no butter or turtle fat	— 0.2	Fasting 2½ hours	0.0860 0.1186	0.0889 0.0412	0.1749 0.1598	Clear, bright, no haemolysis Very slightly opalescent	Non-sterol unsap. matter 0.2554 % 0.1380 %

9	26. iii. 1927	J. A. G.	—	Fasting 4½ hours	0.0671 0.0732	0.1500 0.1437	0.2261 0.2169	Plasma orange and very slightly opalescent Opalescent and opaque, very slight haemolysis		
			330 g. lean beefsteak, 220 g. potato, 110 g. brown bread, 42 g. butter, 56 g. cheese, 1 green artichoke, 1 pint beer	—	—	—	—	—	Non-sterol unsep. matter 0.3504 % 0.3086 %	
10	9. ii. 1928	J. A. G.	—	Fasting 3½ hours	0.0654 0.0671	0.1405 0.1328	0.2069 0.2000	Plasma clear, no haemolysis Milky opalescence, no haemolysis		
			220 g. mutton chop, 2 eggs, Brussels sprouts, 10 g. brown bread, 1 small pancake, 1 pint beer	—	—	—	—	—		
11	22. ii. 1927	J. A. G.	—	Fasting 2 hours 4½ hours	0.0707 0.0776 0.0772	0.0691 0.1234 0.1344	0.1698 0.2010 0.2116	Brownish red, very slight haemolysis Very milky, very slight haemolysis Milky, no haemolysis	Total solids of plasma 9.086 % 9.086 % Heparin used as anticoagulant	
			Borfil, roll, butter, chop, potatoes, Brussels sprouts, 2 scrambled eggs on toast, coffee	—	—	—	—	—		
12	20. vii. 1926	H. G.	—	Fasting 3 hours	0.0601 0.0612	0.1430 0.1318	0.2021 0.1930	Clear yellow, no haemolysis Opalescent, no haemolysis		
			330 g. veal cutlet, potatoes, bread, 56 g. butter, mushroom omelette (3 eggs), coffee with milk	—	—	—	—	—		
13	22. iii. 1927	J. A. G.	—	Fasting 4 hours	0.0708 0.0675	0.1057 0.1325	0.1765 0.2000	Clear brownish, no haemolysis More opalescent, no haemolysis	Heparin used as anticoagulant	
			450 g. potatoes, 180 g. calves' brains, 60 g. beefsteak, 1 egg, 3 slices brown bread and butter, 1 pint beer	—	—	—	—	—		
<i>Some pathological cases.</i>										
14	21. vi. 1925	A. T.	—	Fasting 3 hours	0.0445 0.0410	0.0906 0.0766	0.1411 0.1176	—	Case of Graves' disease	
			Minced meat, vegetable, 1 egg, bread and butter	—	—	—	—	—		
15	25. vii. 1925	A. P.	—	Fasting 2 hours	0.0652 0.0685	0.1071 0.0667	0.1723 0.1562	—	Secondary contracted kidney Red corpuscles 2,720,000 before 2,240,000 after	
			Ordinary hospital dinner	—	—	—	—	—		

to the ester cholesterol; but in Exp. 2 there was also a decrease in free cholesterol. In the next six experiments the meals contained between 0.2 and 0.5 g. of sterol. Here the results are markedly varied. Exps. 4 and 5 show a definite decrease of total cholesterol  $2\frac{1}{2}$  to  $2\frac{3}{4}$  hours after the meal, and this is shared by both free and ester cholesterol. In Exp. 6 there is a small increase of total cholesterol during digestion, mainly due to ester, and in Exp. 9 a decrease, also chiefly in the ester cholesterol.

In Exps. 7 and 8 there is a small change in total cholesterol, in opposite directions in the two experiments, but in both there is a very marked disturbance in the ratio of free to ester cholesterol during the progress of digestion. In both experiments there is a marked increase in the free cholesterol and a corresponding and almost proportional decrease in the ester cholesterol. The two subjects of this experiment lunched together and had similar food, the weights of the various items being as nearly equal as the *chef* could manage. The meal differed from the others of the group in containing very little fat beyond that naturally contained in the lean meat and vegetables. G. E. omitted even the small quantity of butter and left the turtle fat. It seems probable that this deficiency of fat relatively to protein and carbohydrates may be the cause—direct or indirect—of the disturbance in the normal free cholesterol to ester cholesterol ratio.

The meals in Exps. 10–13 were characterised by a relatively high content of sterol. This was effected in Exps. 10, 11 and 12 by the use of eggs and butter, and in Exp. 13 by the addition of calves' brain. In these experiments there was evidently little or no difference between the values for free cholesterol in fasting conditions and during the third to fourth hour of digestion. Any small changes occur chiefly in the ester and are in some cases positive and in others negative.

From a consideration of the whole figures in this table, it is clear that there is no direct connection between the intake of sterol with the food and the sterol content of the blood plasma at the height of digestion. There is therefore no such thing as alimentary hypercholesterolaemia in the usually accepted sense of the term. Since the free and ester cholesterol respectively of the plasma undergo changes during digestion differing both in degree and in direction, it is obvious that these variations cannot be accounted for by any alteration in the plasma dilution.

Our data, which in general confirm the conflicting observations of other workers quoted above, whatever the precise explanation may be of individual cases, seem to indicate that, during digestion, there is an active endogenous metabolism going on, in which the sterols play a part, so that at any moment the cholesterol and cholesterol ester percentages in the blood are the resultant of various factors, just as in the case of any other metabolic products, for example, the purines. As to what these factors are which govern the resultant percentage we have little or no information at present.

In Part II [1927, 2] of this series we brought forward evidence that in the

living plasma cholesterol and its esters are not merely present in simple colloidal solution or suspension, but are in intimate association with the proteins, particularly the euglobulins.

In some of the experiments in the present paper we attempted to correlate changes in the albumin-globulin ratio during digestion with the changes in sterol content, using the refractometric method of Robertson. This method is, of course, open to the objection that a complete separation of albumin and globulin cannot be obtained by a single precipitation with ammonium sulphate, but, apart from this, the method, in our hands at any rate, proved unreliable in the case of some of the milky plasmas taken during digestion.

In recent years the experiments of Gardner and Fox [1921, 1925], Thannhauser [1923], Beumer and Lehmann [1923], Channon [1925] and Randles and Knudson [1925] have proved that cholesterol can be synthesised in the animal organism, but from what source there is no evidence to form any opinion. On the other hand, no properly characterised degradation products of the sterols have, to our knowledge, ever been isolated from the tissues. The close relationship between cholesterol and the bile acids, conclusively proved from different points of view by Windaus and Neukirchen [1919], and by Wieland and Jacobi [1926], is significant, but this similarity of the carbon structure of the molecule in itself scarcely warrants the conclusion that has been drawn, that the bile acids originate through some oxidative breakdown of the sterol molecule. Conclusive evidence on this point can scarcely be obtained until some reliable quantitative method has been discovered for estimating the bile acids.

The unsaponifiable matter of the "lipoid" extract of plasma contains a non-sterol portion, often much greater in amount than the sterols. This non-sterol portion does not give the sterol colour reactions. In chloroform solution it gives with acetic anhydride and a few drops of sulphuric acid only a brownish coloration. This affords a satisfactory test that precipitation of the sterol digitonide has been complete in the estimation of sterol esters, and in the analyses in this paper was always applied. In some cases the non-sterol residue was weighed and the percentage is given in Table II. We make no comment on the figures at this stage, as this residue is not a single substance but a more or less complex mixture. Its composition is under investigation and we hope to be able to communicate the results of our work in the near future.

There is a further point of interest in the figures of Table II which is perhaps worth mention, namely, the constancy of the fasting values for J. A. G., which extend over a period of 3 years. The free cholesterol figures are of much the same order, the lowest being that obtained 3 years ago. Some variations occur in the ester cholesterol, but these are small compared with those observed during digestion.

From our results it would appear advisable, in making comparative determinations of sterols in pathological cases, to work with blood from patients in a fasting condition, or at any rate after only a very light meal.

## SUMMARY.

1. The level of the cholesterol content of human plasma, taken while fasting, can be raised or lowered by sufficiently prolonged feeding with diets of high or low sterol content. Such changes are most marked as regards the cholesterol in ester form; the free cholesterol remains practically constant.

2. As the result of a single meal, alimentary hypercholesterolaemia does not occur, and there is no connection between the amount of sterol ingested and the cholesterol level of the plasma during digestion.

3. During the process of digestion changes in the cholesterol content of the plasma frequently occur, sometimes an increase, sometimes a decrease compared with fasting values, and under certain conditions a marked disturbance of the free to ester cholesterol ratio is observed.

We consider these changes evidence of an active endogenous metabolism, in which cholesterol takes part, during the process of digestion.

4. It is important, in comparing the cholesterol content of plasma under normal and pathological conditions, to use bloods drawn while the subject is in a fasting condition.

We take this opportunity of thanking the Medical Research Council for a grant to one of us, by which the expenses of this investigation were in part defrayed.

## REFERENCES.

- Beumer and Lehmann (1923). *Z. ges. exp. Med.* **37**, 274.  
Bürger and Habs (1927). *Z. ges. exp. Med.* **56**, 640.  
Channon (1925). *Biochem. J.* **19**, 424.  
Denis (1917). *J. Biol. Chem.* **29**, 93.  
Dorée and Gardner (1908). *Proc. Roy. Soc. Lond. B*, **80**, 230.  
Gardner and Fox (1921). *Proc. Roy. Soc. Lond. B*, **92**, 358.  
——— (1925). *Proc. Roy. Soc. Lond. B*, **98**, 76.  
Gardner and Gainsborough (1927, 1). *Biochem. J.* **21**, 130.  
——— (1927, 2). *Biochem. J.* **21**, 141.  
Grigaut (1913). *Le cycle de cholestérimie* (G. Steinheil, Paris), p. 191.  
Luden (1917). *J. Lab. Clin. Med.* **3**, 141.  
Müller (1915). *J. Biol. Chem.* **22**, 1.  
——— (1916). *J. Biol. Chem.* **27**, 451.  
Randles and Knudson (1925). *J. Biol. Chem.* **66**, 459.  
Rouzaud and Cabanis (1913). *Compt. Rend. Soc. Biol.* **74**, 469.  
Thannhauser (1923). *Z. physiol. Chem.* **127**, 278.  
Widal, Weill and Laudat (1912). *Semaine médic.* **32**, 529.  
Wieland and Jacobi (1926). *Ber. deutsch. chem. Ges.* **59**, 2064.  
Wieland and Sorge (1916). *Z. physiol. Chem.* **87**, 1.  
Windaus and Neukirch (1919). *Ber. deutsch. chem. Ges.* **52**, 1915.

## CXXX. ON THE PIGMENT OF THE FAT OF CERTAIN RABBITS.

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*(Received June 22nd, 1928.)*

ALTHOUGH the adipose tissue of man and certain farm animals is characterised by the presence of varying amounts of pigment, in certain other species of mammals, such as rodents, it appears to be almost entirely absent. Reference to the literature confirms this view [Palmer, 1922, p. 132], although Lewkowitsch [1922] states that rabbit fat is of a "dirty yellow colour." It was therefore both interesting and surprising to find, in the course of certain studies on genetics by other workers [Pease, 1928], that occasionally rabbits do exhibit adipose tissues that are deeply pigmented. Moreover, Pease has shown that this fat pigmentation follows the Mendelian laws and has demonstrated the existence of two types, one having yellow fat and the other white. The heterozygous animal has white fat, so that the white factor is the dominant.

From the biochemical point of view two problems suggested themselves for further study, namely, the nature of the colouring matter in the "pigmented" rabbit, and the reasons for its absence from the "unpigmented" rabbit. Only the former problem was investigated here and somewhat unexpectedly [*vide* Palmer, 1914] the pigment present proved to be xanthophyll, derived from the food. As it was not possible to carry the investigation further it was decided to publish the results obtained. The latter problem has since been investigated by Bernheim [1928]. He confirmed the presence of xanthophyll in the fat of the "pigmented" rabbit and found that its absence from the "non-pigmented" rabbit was due, not to an inability to absorb the pigment, but to the fact of its oxidation in the liver by a thermolabile system. This system was apparently absent from, or inhibited in, the liver of yellow-fatted rabbits.

### EXPERIMENTAL.

The adipose tissue from a "pigmented" male rabbit (*Lepus cuniculus*) was carefully dissected off yielding in all some 175 g. The animal had been fed on the usual diet of bran, oats, hay and cabbage leaves without restriction. The character of the food is important because the pigment will only appear in the "pigmented" rabbit when some greenstuff is included in its rations. On the other hand, no matter what food is fed, the "non-pigmented" animal never develops pigment. In the case of the "pigmented" animal the food supply is thus the source of the pigment.

The fatty tissue was minced three times giving 170 g. of material with a colour of about the same intensity as that of New Zealand butter. It was then rendered in an oven at 65°, 134 g. of clear melted fat being decanted. The Sp. Gr. of the rendered fat was 0.930 (15.5°). The residue consisted of darker-coloured material, part of which was obviously tissue other than fat, and this was rejected. The pigmented fat was then immediately saponified with colourless 20 % alcoholic potash, using 2 cc. per g. of fat. In order to avoid the formation of resins the ethyl-alcohol employed in the preparation of the alcoholic potash had been carefully purified by treatment with silver nitrate and unslaked lime. After 1 hour's gentle boiling under reflux the solution was cooled and diluted and the pigment extracted with purified ether in the usual way. The ethereal extract, which was deep yellow in colour, was then washed with distilled water until the washings no longer reacted alkaline to phenolphthalein. The washed extract was then dried over anhydrous sodium sulphate and the solvent removed under reduced pressure since the pigment is readily oxidised in ether. A residue of clean orange globules remained and these were dissolved in pure carbon disulphide and kept in the dark, and a portion was reserved for chromatographic analysis.

The unsaponifiable matter was readily soluble in the usual fat solvents (light petroleum, chloroform, etc.) giving yellow-coloured solutions. In carbon disulphide an orange solution was obtained, whilst in formic acid a green tint was noticeable on dilution. With antimony trichloride a slaty-blue colour was obtained. A portion of the unsaponifiable matter was taken up in 80 % alcohol and shaken with light petroleum (B.P. 50–60°) in the phase test. No pigment was given up to the light petroleum layer. These results suggested that the colouring matter present was probably xanthophyll.

The chromatographic analysis of Tswett [1906] provides another method of distinguishing xanthophyll pigments from carotin. Calcium carbonate, previously dried for several hours at 150°, was used as adsorbent. A glass adsorption tube of 2 cm. diameter and 12 cm. length was drawn out to a point at one end so as to leave an orifice of 2 mm. diameter. The narrow end was then closed by a tight-fitting plug of cotton-wool which was followed by the dry calcium carbonate. Great care was taken to fill the tube as evenly and tightly as possible. It was then connected with a small filter flask, suction applied and a stream of pure carbon disulphide passed through the column. A little of the pigment solution in carbon disulphide was now poured into the tube with gentle suction. This was followed by more of the pure solvent so as to establish a stream through the adsorbing column. Different adsorption rings were observed but the largest and best defined was of light orange colour and presumably represented xanthophyll *a*. No evidence was obtained for the presence of carotin. It was concluded therefore that xanthophyll pigments must be present.

Parallel with the saponification of the pigmented fat, a control experiment was carried out with the fat from an "unpigmented" male rabbit of about the

same weight as the "pigmented" animal. 145 g. of white fat were saponified. The unsaponifiable matter was isolated in exactly the same way as for the pigmented fat already considered. The dried ethereal extract containing the unsaponifiable matter was practically devoid of colour, and on removal of the solvent the solid matter was only faintly pigmented. Carotinoid pigments were also found to be absent when the blood-serum of the same rabbit was examined by Palmer's method [1922, p. 207]. These results showed that the colouring matter of the pigmented fat could not have been developed during the saponification process but was derived from the animal's tissues.

#### SUMMARY.

1. Certain rabbits exhibit deeply pigmented adipose tissues.
2. The colouring matter present has been identified as consisting of xanthophyll pigments.

I am indebted to Mr M. S. Pease for the material employed and for information as to the feeding of the animals.

#### REFERENCES.

- Bernheim (1928). Private communication.  
Lewkowitsch (1922). *Oils, fats and waxes*, 2 (6th edition, London), p. 691.  
Palmer (1914). *J. Biol. Chem.* 17, 191.  
—— (1922). *Carotinoids and related pigments* (New York).  
Pease (1928). *Proc. 5th Internat. Congr. Genet.* 2.  
Tswett (1906). *Ber. deutsch. botan. Ges.* 24, 316.



# CXXXI. PANCREATIC EXTRACTS IN RELATION TO LACTIC ACID FORMATION IN MUSCLE.

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THE pancreatic factor of Winfield and Hopkins [1915] which inhibits the formation of lactic acid in muscle and muscle extracts, has been shown [McCullagh, 1928] to owe its effect to suppression of esterification of phosphates. The first portion of the work herein reported was carried out, using a new technique to be described, with a view to discovering the properties of the pancreatic factor and its distribution throughout various body tissues. Results were soon obtained, however, which led us to suspect the conclusions of former workers in regard to the possible identity of this factor with other tissue constituents. It seems to have been established that neither trypsin nor insulin is responsible for the inhibition; and Downes [1927], Reay [1927] and Ronzoni [1927] state that in their opinion the inhibitory effect of pancreas and pancreatic extracts is not due to amylase. Our preliminary results, however, indicated the necessity of further work in this connection.

## PART I. PROPERTIES AND DISTRIBUTION OF THE FACTOR.

*Methods.* Preparation of the muscle extract was at first carried out as described in the previous paper. However, it was speedily found that such elaborate precautions in regard to temperature were superfluous; the extracts obtained by the following procedure are, in general, if anything more active. Immediately after amytal anaesthesia the rabbit is bled from the heart, the hind quarters are at once stripped and the leg muscles removed. Mincing is followed by extraction with three times the weight of ice-cold distilled water for half an hour, the extracting vessel being kept immersed in a freezing mixture. After filtering through muslin the filtrate is kept cold in the same way, and apart from this no cooling measures are necessary.

### *Technique employed for the study of the pancreatic factor.*

In the presence of fluoride, hexosephosphate is not hydrolysed by muscle enzymes and free phosphate therefore rapidly disappears owing to its removal by esterification. As formerly shown the free phosphate does not disappear

in the presence of the pancreatic extract. The new method of studying the pancreatic inhibitor is based on the measurement of the suppression of esterification of phosphate in the presence of fluoride.

The following substances are placed in a test-tube:

(a) 1 cc. extract containing the pancreatic factor (or distilled water for control);

(b) 2 cc. 2 % soluble starch;

(c) 2 cc. fluoride buffer (as described in former work);

(d) 5 cc. muscle extract.

The contents of the tubes are thoroughly mixed and a 3 cc. sample is immediately withdrawn and delivered into a test-tube containing 10 cc. 6 % trichloroacetic acid. The mixture is then incubated for 1 hour at 30° and again sampled in the same manner. The samples are thoroughly mixed with the acid, allowed to stand 5 minutes and filtered. Free phosphate is estimated in 3 cc. of the filtrate by the Briggs modification of the Bell-Doisy method.

In studying the distribution of the factor in various tissues, the extract was prepared in each case by triturating the tissue with sand plus ten times its weight of water, allowing it to stand in the ice-chest for 1 hour previous to centrifuging, and using the supernatant fluid.

#### *Some properties of the inhibiting factor.*

*Thermostability.* The statements in the literature concerning the thermostability of the inhibiting factor vary somewhat. 5 cc. of pancreatic extract were placed in a thin glass test-tube which was immersed in a boiling water-bath for 2 minutes. This caused a complete destruction of the inhibiting factor as shown by Table I.

Table I.

	mg. lactic acid in 15 cc. sample.		
	Before incubation	After incubation	Change
1. Water control	9.7	25.8	16.1
2. Pancreas	9.7	10.3	0.6
3. Boiled pancreas	9.7	25.7	16.0

In this experiment the technique of the former paper was followed, viz. each flask contained 25 cc. muscle extract, 10 cc. 2 % starch, 10 cc. phosphate buffer as previously described, and 5 cc. pancreatic extract or water. The production of lactic acid during an hour's incubation was used as a criterion of activity. The slight increase in lactic acid in the flask containing unboiled pancreas was probably derived from preformed hexosephosphate.

*Dialysability.* As a result of several attempts we have found that the inhibiting factor will not pass through a collodion membrane. The formation of hexosephosphates was in no case suppressed by the addition of the outer dialysing fluid to the incubation mixture, whereas the inner dialysing fluid was at the same time very active. Table II gives typical results, dialysis in this case being continued for a week.

Table II.

mg. P as free phosphate in 3 cc. sample.			
	Before incubation	After incubation	Change
1. Water control	0.81	0.32	- 0.49
2. Inner fluid	0.80	0.80	0.00
3. Outer fluid	0.80	0.32	- 0.48

*Adsorbability.* Reay and Downes made several attempts to adsorb the factor with but little success; in view of other work to be reported it seemed unnecessary to confirm these results. We have, however, carried out experiments in regard to the precipitability of the factor by means of heavy metals, etc., but have obtained only negative results.

*Distribution of the inhibiting factor.*

Pancreas preparations were always found to be 50 to 500 times as efficacious as any other preparations except those from tryptic juice, saliva, and salivary glands. Table III gives figures showing the effect of various tissues on the formation of hexosephosphate.

Table III.

mg. P as free phosphate in 3 cc. sample.				
	Before incubation	After incubation	Change	% inhibition
1. Water control	0.84	0.16	- 0.68	0
2. Pancreas (ox, no. 1)	0.86	0.84	- 0.02	97
3. Pancreas (ox, no. 2)	0.85	0.36	- 0.49	28
4. Parotid (rabbit)	0.89	0.89	0.00	100
5. Kidney (rabbit)	0.85	0.31	- 0.54	21
6. Liver (rabbit)	0.86	0.48	- 0.38	44
7. Suprarenals (rabbit)	0.86	0.32	- 0.54	21
8. Spleen (rabbit)	0.87	0.46	- 0.41	40
9. Brain (rabbit)	0.84	0.23	- 0.61	10
10. Bladder (rabbit)	0.85	0.21	- 0.64	6
11. Leg muscle (rabbit)	0.86	0.16	- 0.70	3 % acceleration
12. Ovary (rabbit)	0.85	0.18	- 0.67	1
13. Lung (rabbit)	0.85	0.23	- 0.62	9
14. Kidney (ox)	0.84	0.34	- 0.50	26
15. Liver (ox)	0.85	0.56	- 0.29	57
16. Spleen (ox)	0.84	0.45	- 0.39	43
17. Brain (ox)	0.85	0.26	- 0.59	13
18. Bladder (ox)	0.85	0.21	- 0.64	6
19. Lung (ox)	0.84	0.28	- 0.56	18

All the above preparations are aqueous extracts. With the exception of the pancreas all are prepared so that 1 g. of tissue is equivalent to 10 cc. of extract. In the case of pancreas (ox, no. 1) 1 g. represents 25 cc., while in the case of pancreas (ox, no. 2) 1 g. represents 1.25 l.

All tissues except parotid show an inhibition of the same order as the very dilute pancreas preparation. Unreported experiments show inhibitions between 15 and 80 % to be in almost direct proportion to the amount of inhibitor present.

Table IV is the protocol of an experiment to show the relative concentrations of inhibitor in saliva, tryptic juice, and pancreas.

Table IV.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition
1. Water control	0.84	0.38	-0.46	0
2. Saliva 1 : 25	0.86	0.81	-0.05	89
3. Saliva 1 : 100	0.86	0.67	-0.19	59
4. Saliva 1 : 500	0.86	0.49	-0.37	20
5. Tryptic juice 1 : 50	0.85	0.84	-0.01	98
6. Tryptic juice 1 : 100	0.85	0.77	-0.08	82
7. Tryptic juice 1 : 500	0.85	0.60	-0.15	67
8. Tryptic juice 1 : 1000	0.85	0.48	-0.37	20
9. Pancreas 1 : 250	0.85	0.74	-0.11	76
10. Pancreas 1 : 500	0.85	0.60	-0.25	56
11. Pancreas 1 : 1000	0.86	0.43	-0.43	7

From this it is evident that these tissues and fluids all possess approximately the same power to inhibit the formation of hexosephosphate, 1 cc. of a 1 in 500 dilution of saliva being slightly less potent than the 1 in 500 tryptic juice and the 1 in 500 pancreas preparation.

From the results of the foregoing experiments concerning the properties and distribution of the inhibitor it was noted that:

- (a) it is not more thermostable than amylase;
- (b) like amylase it will not pass through a dialysing membrane;
- (c) it resembles amylase in being difficult to adsorb;
- (d) the distribution corresponds to that of amylase.

This led us to suspect that the phenomenon was nothing more than a manifestation of amylolytic activity. Part II of this paper reports work which confirms this view.

## PART II.

The first point which presented itself was whether or not the inhibition might arise from the muscle enzyme system being destroyed or inactivated by the pancreas. This point was settled by the following experiment, the results of which are given in Table V. In tubes (1) and (2) muscle extract and pancreas were incubated together for 15 minutes at 30°, while in tubes (3), (4) and (5) the muscle extract was simultaneously incubated alone. At the end of this primary incubation the remainder of the substances (starch, etc.) was added to all the tubes and the initial samples were withdrawn. Incubation was then carried on as usual for 1 hour at 30°. The pancreas preparation used was an aqueous extract of ox pancreas, 250 cc. of which represented 1 g. of pancreas, 1 cc. being used in each tube as usual. A preliminary experiment had shown that this extract would bring about only a partial inhibition with respect to the muscle enzyme used.

Table V.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition
1. Pancreas	0.87	0.71	-0.16	71
2. Pancreas	0.86	0.70	-0.16	71
3. Pancreas	0.84	0.67	-0.17	70
4. Pancreas	0.83	0.67	-0.16	71
5. Water control	0.84	0.33	-0.51	0

The incubation of numbers 1 and 2 with pancreas has made no appreciable difference in the amount of inhibition. It is probable that the slight excess of phosphate present in tubes 1 and 2 was derived from hexosephosphates during the first incubation. During this period the resynthesis of hexosephosphate from glycogen and free phosphate would not take place extensively owing to the presence of pancreas. In tubes 3 and 4 there would be some synthesis of hexosephosphate during the primary incubation. Tube 5 was the water control. This demonstrates that the muscle enzyme is not being destroyed by the pancreatic extract.

The extent of inhibition, if due to an amylolytic effect on the starch, should decrease if the percentage of starch in the incubation mixture is increased. After preliminary experiments to ascertain the amount of pancreas necessary to give a partial inhibition with the muscle enzyme preparation in use, the following experiments were performed to obtain information on this point.

Table VI.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition
1. H <sub>2</sub> O and 2 % starch	0.68	0.12	- 0.56	0
2. Pancreas and 2 % starch	0.68	0.60	- 0.02	96
3. H <sub>2</sub> O and 4 % starch	0.68	0.12	- 0.56	0
4. Pancreas and 4 % starch	0.68	0.32	- 0.36	36
5. H <sub>2</sub> O and 6 % starch	0.68	0.12	- 0.56	0
6. Pancreas and 6 % starch	0.68	0.22	- 0.46	18

From this it appears that an increase in starch concentration within the above limits does not affect the rate of formation of hexosephosphate by the muscle enzyme system. The increasing amounts of substrate, however, do cause a marked decrease in potency of the pancreatic inhibitor. This indicates that the inhibition must be due, at least in part, to amylase.

Wohl and Glimm [1910] have shown that the activity of amylase is suppressed in the presence of large amounts of maltose, whereas sucrose, galactose, and fructose have no effect on the amylolytic activity. We find that, in the presence of large amounts of sugar the muscle enzyme system is less active. The pancreas, however, still causes its usual inhibition except in the presence of maltose. Table VII is the protocol of an experiment to demonstrate this.

Table VII.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition due to pancreas
1. Water control	0.66	0.05	- 0.61	0
2. Pancreas	0.67	0.68	+ 0.01	101
3. H <sub>2</sub> O and 2 g. galactose	0.60	0.28	- 0.32	0
4. Pancreas and 2 g. galactose	0.60	0.60	0.00	100
5. H <sub>2</sub> O and 2 g. sucrose	0.60	0.05	- 0.55	0
6. Pancreas and 2 g. sucrose	0.61	0.60	- 0.01	99
7. H <sub>2</sub> O and 2 g. fructose	0.59	0.25	- 0.34	0
8. Pancreas and 2 g. fructose	0.59	0.60	+ 0.01	101
9. H <sub>2</sub> O and 1.5 g. maltose	0.62	0.21	- 0.41	0
10. Pancreas and 1.5 g. maltose	0.62	0.44	- 0.18	56

Tube no. 1 is a water control without any pancreas or sugar. Another water control was incubated with each of the sugars used, as the different sugars varied in the degree in which they themselves inhibited the muscle enzyme system. In each case much more pancreas was used than was necessary to cause complete inhibition. The sugars were added in crystalline form to the incubation mixtures.

Percentage inhibitions by pancreas in the above table are calculated, in each case, by considering the corresponding tube containing sugar alone to show no inhibition. In the presence of galactose, sucrose, or fructose, the pancreas causes complete inhibition. In the case of maltose, however, in spite of the large amount of pancreas present there was only a partial inhibition.

The experiment on maltose was repeated using a pancreatic preparation which, by means of previous experiments, we knew to be sufficiently dilute to cause only a partial inhibition in the absence of maltose. Table VIII gives the results.

Table VIII.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition due to pancreas
1. Water control	0.78	0.05	- 0.73	0
2. Pancreas	0.78	0.63	- 0.15	79
3. H <sub>2</sub> O and 1.5 g. maltose	0.71	0.51	- 0.20	0
4. Pancreas and 1.5 g. maltose	0.70	0.50	- 0.20	0

It is evident that with these more dilute solutions of pancreas the maltose is capable of completely preventing activity of the inhibitor.

In both the above experiments there is an apparent discrepancy between the figures given for phosphates previous to incubation. This is caused by the changes in volume produced by the addition of the solid sugars.

If the inhibiting substance is amylase, different amylolytic preparations should have the same relative diastatic power as they have relative power of inhibition. On April 30th, 1928, we had the following preparations in the ice-chest, where they had been stored: (1) an alcoholic preparation of desiccated pancreas prepared March 2nd, 1928; (2) a similar preparation of March 29th, 1928; (3) a preparation which had been subjected to tryptic digestion at 37° for 3 days, previous to removing trypsin and protein with alcohol in the usual manner; (4) an alcoholic preparation of April 26th, 1928; (5) an alcoholic preparation of saliva made on April 26th, 1928.

The inhibiting power of these preparations was now tested. In each case a series of tubes containing the preparation in various dilutions was incubated for 1 hour in the ordinary manner at 30°, with the results shown in Table IX. Only those dilutions which are of interest in demonstrating the relative inhibiting power are given in the table.

Preparations 2 and 4 are the most potent and about equal in inhibiting power. Preparation 5 is next in strength, whereas 1 and 3 are both less effective.

Table IX.

mg. P as free phosphate in 3 cc. sample.

	Before incubation	After incubation	Change	% inhibition
1. Water control	0.72	0.10	- 0.62	0
2. Pancreas prep. 1 (undiluted)	0.72	0.55	- 0.17	77
3. Pancreas prep. 2 dil. 1 : 10	0.73	0.52	- 0.21	66
4. Pancreas prep. 3 (undiluted)	0.73	0.54	- 0.19	69
5. Pancreas prep. 4 dil. 1 : 10	0.74	0.55	- 0.19	69
6. Saliva prep. dil. 1 : 5	0.74	0.42	- 0.32	48

The relative diastatic powers of these same preparations were measured by mixing 1 cc. of each preparation with 5 cc. 1 % soluble starch and 3 cc. of chloride-containing buffer, and then incubating at 30°. In each case the time was noted at which a two-drop sample showed with iodine a change from the pure starch blue colour.

The results are given in the following table:

Table X.

Preparation	1	2	3	4	5
Time in mins.	30	5	40	5	20

Once more preparations 2 and 4 proved to be the strongest, followed by 5, with 1 and 3 the least potent. That is, they have the same relative diastatic activity as they have power of inhibiting esterification.

It seemed desirable to add confirmation by measurement of the power to inhibit the actual production of lactic acid. To this end flasks were set up as follows, and lactic acid determinations carried out before and after incubation. The technique was the same as in the former paper except that the method of Friedemann, Cotonio and Shaffer [1927] was used for the determination of lactic acid. Each flask contained 25 cc. muscle extract, 10 cc. soluble starch, and 10 cc. phosphate buffer. Besides this, flask no. 1 contained 5 cc. water; no. 2, 5 cc. of a 1 in 2 dilution of pancreas preparation 1; no. 3, 5 cc. of a 1 in 20 dilution of preparation 2; no. 4, 5 cc. of a 1 in 2 dilution of preparation 3; no. 5, 5 cc. of a 1 in 20 dilution of preparation 4; no. 6, 5 cc. of a 1 in 10 dilution of saliva preparation no. 5. These dilutions were all calculated to give a partial inhibition. The results of the lactic acid determinations are given in Table XI.

Table XI.

mg. lactic acid in 15 cc. sample.

	Before incubation	After incubation	Change	% inhibition
1.	6.4	23.7	17.3	0
2.	7.0	15.4	8.4	51
3.	6.4	12.2	5.8	66
4.	6.6	14.0	7.4	57
5.	5.8	11.8	6.0	65
6.	5.4	12.2	7.8	55

When the dilutions of the various preparations are considered it is seen that again the distribution of inhibiting power is the same as in the two

previous experiments; preparations 2 and 4 being more potent than 5, which is half as strong, and 1 and 3 which are about one-tenth as strong.

This incidentally demonstrates the admissibility of results obtained by the phosphate technique, as did other lactic acid measurements made at intervals throughout the work.

It seemed possible that, although a part of the inhibition was due to amylase, there might be some other factor present in pancreas which would inhibit esterification. If such a factor be present it is unlikely that it would be destroyed by heat at exactly the same rate as the amylase.

10 cc. samples of a preparation made from desiccated pancreas (as described in the former paper) were placed in test-tubes and immersed in a boiling water-bath for 18 and 21 seconds respectively. The diastatic action of these preparations was tested by the same method as that described above. The times before a change in colour was observed are as follows:

	Original preparation	Sample no. 1	Sample no. 2
Time (mins.)	4	8.25	12

From the above the original preparation is seen to be practically twice as powerfully diastatic as sample no. 1, and three times as strong as no. 2.

These amylolytic solutions were now tested for inhibiting power by setting up tubes in various dilutions. The results obtained with the tubes which contained suitable dilutions of the various solutions are given in Table XII.

Table XII.

mg. P as free phosphate in 3 cc. sample.				
	Before incubation	After incubation	Change	% inhibition
1. Water control	0.72	0.13	- 0.59	0
2. Original pancreas dil. 1 : 6	0.72	0.48	- 0.24	59
3. Sample no. 1 dil. 1 : 3	0.72	0.45	- 0.27	54
4. Sample no. 2 dil. 1 : 2	0.72	0.45	- 0.27	54

This demonstrates that in respect to inhibiting power the original preparation is twice as active as sample no. 1, and three times as active as sample no. 2, which indicates that the only substance in these preparations capable of inhibiting esterification is destroyed by heat at the same rate as amylase.

Meyerhof [1927] has recently pointed out that the muscle enzyme system is not only capable of producing lactic acid from starch, glycogen, and hexosans, but can also produce it from glucose if that sugar be activated. He describes methods of preparing activator from muscle or from yeast. If the inhibiting effect of pancreas on the lactic acid production is a function of amylase, one should not expect inhibition to occur when lactic acid is produced from glucose, even in the presence of pancreas. The activator in the following experiments was prepared from yeast by the method described by Meyerhof. In this experiment six flasks were set up as follows:

1. 25 cc. muscle extract + 10 cc. phosphate buffer + 10 cc. 2 % starch + 5 cc.  $H_2O$ .



2. 25 cc. muscle extract + 10 cc. phosphate buffer + 10 cc. 2 % starch + 5 cc. pancreas.
3. 25 cc. muscle extract + 10 cc. phosphate buffer + 15 cc.  $H_2O$ .
4. 25 cc. muscle extract + 10 cc. phosphate buffer + 5 cc. 4 % glucose + 10 cc.  $H_2O$ .
5. 25 cc. muscle extract + 10 cc. phosphate buffer + 5 cc. 4 % glucose + 5 cc.  $H_2O$  + 5 cc. activator.
6. 25 cc. muscle extract + 10 cc. phosphate buffer + 5 cc. 4 % glucose + 5 cc. pancreas + 5 cc. activator.

Lactic acid estimations were carried out before and after incubation and Table XIII gives the results.

Table XIII.

mg. lactic acid in 15 cc. sample.			
	Before incubation	After incubation	Change
1.	10.9	19.3	8.4
2.	11.9	14.9	3.0
3.	10.8	12.8	2.0
4.	10.4	12.8	2.4
5.	10.8	27.2	16.4
6.	13.1	32.0	18.9

Flasks 1 and 2 show that both the muscle enzyme preparation and the pancreas are quite active. The lactic acid formation from glycogen and hexose-phosphate in the muscle extract is small as demonstrated by flask 3. Flask 4 shows that the addition of glucose causes no increase in the amount of lactic acid formed, unless, as in flask 5, activator be added. Flask 6 makes it quite evident that the formation of lactic acid from activated glucose is not affected by the presence of pancreas.

It has been shown above that animal amylases have the power of inhibiting the formation of hexosephosphates, and *ipso facto*, that of lactic acid from starch. If this is a purely amylolytic phenomenon, plant diastases would be expected to have the same effect. We therefore tested takadiastase and malt diastase, with the results given in Table XIV.

Table XIV.

mg. P as free phosphate in 3 cc. sample.				
	Before incubation	After incubation	Change	% inhibition
1. 1 cc. $H_2O$	0.78	0.08	-0.70	0
1 cc. takadiastase	1.52	1.51	-0.01	99
2. 1 cc. $H_2O$	0.66	0.05	-0.61	0
1 cc. malt diastase	0.68	0.50	-0.18	70

Controls with boiled enzymes showed no inhibition. The high values for takadiastase are due to the phosphate content of the commercial preparation.

As expected, both these preparations are quite active as inhibitors. The malt diastase did not cause complete inhibition, but was not a highly active diastatic preparation.

As starch has been used as a substrate in nearly all the work herein recorded, it seemed advisable to show that the inhibition also occurs when the substrate is glycogen. Using a 2 % solution of glycogen instead of the usual 2 % soluble starch the figures given in Table XV were obtained.

Table XV.

	mg. P as free phosphate in 3 cc. sample.			
	Before incubation	After incubation	Change	% inhibition
1. Water control	0.84	0.29	0.55	0
2. Pancreas	0.84	0.84	0.00	100

The inhibition therefore occurs whether starch or glycogen is used.

In many of the above experiments the mixture was tested with iodine immediately before incubation. Starch was found to be present, even in tubes where inhibition proved to be complete. This eliminates the possibility that starch has been removed by enzymic hydrolysis before it becomes accessible to the muscle enzymes.

In all the experimental work reported above, guarded pipettes were always employed, in order to avoid the possibility of salivary contamination.

#### DISCUSSION AND SUMMARY.

The first part of the paper demonstrates that the properties and distribution of the factor are the same as those of amylase. It is shown in the second part of the paper that the muscle enzyme system is not itself affected by the inhibitor. As different concentrations of substrate cause variation in the inhibiting power of pancreas, the action would appear to be on the starch. Amylase is inhibited in its activity by maltose but not by certain other sugars; this is also the case with the pancreatic factor. The amylolytic power and the ability to inhibit esterification and lactic fermentation have been shown to correspond in the case of different preparations, and to be destroyed to the same extent by heat. The production of lactic acid from glucose is not in the least inhibited by the pancreas. Both animal and vegetable amylases act as inhibitors when the substrate is starch or glycogen.

These results all indicate that the so-called pancreatic factor which inhibits lactic acid production by muscle enzymes is merely amylase. Statements in the literature to the effect that preparations diastatically active cause no inhibition are probably explained by the fact that the workers in question employed muscle hash, in which partial inhibitions are much more difficult to measure. The same fact undoubtedly accounts for their failure to obtain complete inhibitions. Not only does the question of permeability come into play, but the presence of an activating mechanism in muscle hash would probably result in the production of considerable amounts of lactic acid from glucose, even in the presence of pancreas. Using the muscle enzyme system of Meyerhof, we have never succeeded in obtaining either a preparation containing the inhibiting factor which was not diastatically active or *vice versa*.

Dakin and Dudley [1913] have demonstrated the presence of an anti-glyoxalase in pancreas. Ariyama [1928] has shown the production under certain circumstances of a glyoxal-like compound from hexosephosphate by muscle enzymes. After the destruction of glyoxalase by heat or by means of anti-glyoxalase, the best yield of this substance obtained was only 11 % of the theoretical amount. Thus antiglyoxalase might conceivably cause a partial inhibition. It is our opinion that other than antiglyoxalase and amylase there is no substance in pancreas which inhibits the formation of lactic acid in muscle.

The observations and conclusions recorded above render slight the possibility that some specific pancreatic factor controls lactic acid production in muscle. Apart from any physiological significance, however, the results are of great interest from a purely biochemical standpoint. It is possible that this work may open up a new path whereby a study may be made of the carbohydrate precursor of hexosephosphates in muscle. Moreover, in view of the relatively small amounts of pancreas which are capable of bringing about complete and instantaneous inhibition, it may be that some molecular union between amylase and its substrate, prior to enzymic hydrolysis, can be demonstrated.

Our best thanks are due to Sir F. G. Hopkins and Mr J. B. S. Haldane for their interest and advice during this work.

#### REFERENCES.

- Ariyama (1928). *J. Biol. Chem.* **77**, 395.  
Dakin and Dudley (1913). *J. Biol. Chem.* **15**, 463.  
Downes (1927). Thesis (Cambridge).  
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.  
McCullagh (1928). *Biochem. J.* **22**, 402.  
Meyerhof (1927). *Biochem. Z.* **183**, 176.  
Reay (1927). Thesis (Cambridge).  
Ronzoni (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 178.  
Winfield and Hopkins (1915). *J. Physiol.* **50**, Proc. v.  
Wohl and Glimm (1910). *Biochem. Z.* **27**, 349.

# CXXXII. ON THE APPLICATION OF THE DONNAN EQUILIBRIUM TO THE IONIC RELATIONS OF PLANT TISSUES.

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(Received June 22nd, 1928.)

## INTRODUCTION.

It is a matter of general knowledge that, if a plant tissue is placed in a solution of an electrolyte the concentration of which is greater than its concentration in the tissue, the cation and anion are absorbed in different amounts, electrostatic neutrality being preserved by the outward diffusion of other ions from the tissue. The mechanism controlling this unequal intake of ions is one of which we are still in comparative ignorance; but it has been suggested that the interpretation may be associated with the establishment of a Donnan membrane equilibrium between the cells of the tissue and the external solution. It is the purpose of this paper to inquire more closely into the possibility of this suggestion being correct, and into the extent to which other factors may be associated with the ionic equilibria of the living cell.

## THE INADEQUACY OF A SIMPLE DONNAN SYSTEM.

If two electrolytes in solution are separated by a membrane which is impermeable to one of the ions of one of the electrolytes, the diffusible ions proceed to an equilibrium at which they are unequally distributed on the two sides of the membrane, but at which the product of the concentrations of each pair of oppositely charged diffusible ions is the same on either side; these products will be equal whether only a single electrolyte is concerned or whether there is a mixture of a number. It is this condition which is known as the Donnan equilibrium.

The distribution of an electrolyte in such a system will be expressed by the following equation<sup>1</sup>:

$$\frac{x_i y_i}{v_i^2} = \frac{x_e y_e}{v_e^2}; \quad \dots\dots(1)$$

where  $x_i$  and  $y_i$  represent the amounts of cation and anion respectively within the membrane at equilibrium,  $x_e$  and  $y_e$  represent the equilibrium amounts of the same pair of ions in the external solution, and  $v_i$  and  $v_e$  denote the

<sup>1</sup> If the valency of the  $x$  ions were  $m$  and that of the  $y$  ions  $n$  then we should have  $x^n y^m$  instead of  $xy$ —the special case for univalent ions. We have limited ourselves to the consideration of univalent ions but our arguments can easily be extended to other cases.

volume of the solvent on either side of the membrane. It is clear that whatever the concentration of indiffusible ions within the membrane the above product equation must hold, since it is derived from the fundamental laws of thermodynamics: and, if there are no indiffusible ions in the system at all, the individual ionic concentrations will all be equal; *i.e.*

$$\frac{x_i}{v_i} = \frac{y_i}{v_i} = \frac{x_e}{v_e} = \frac{y_e}{v_e}.$$

Unfortunately experimental verification of the product law is difficult in the case of ordinary plant tissue<sup>1</sup>, owing to the impracticability of estimating  $x_i, y_i$  and  $v_i$ : while the net amount of ions absorbed from a solution could be calculated from the difference between their concentration before putting in the tissue and after equilibrium had been reached, this would not allow for the amounts of these ions which in many cases would almost certainly be resident in the tissue at the commencement of the experiment.

If we are dealing with a simple Donnan system we shall have the following equation:

$$\frac{(X+x)(Y+y)}{v_i^2} = P, \quad \dots\dots(2)$$

wherein  $X$  and  $Y$  denote the amounts of the cation and anion respectively present in the tissue,  $x$  and  $y$  denote the net amounts of the same ions absorbed from the external solution,  $v_i$  has the same significance as in equation (1), and  $P$  is the product of the concentrations of the two ions in the external solution at equilibrium.

A study has recently been made by Petrie [1927] of the variation with temperature in the uptake of the two ions of single salts by carrot-root tissue<sup>2</sup>. The results of this inquiry provide convenient data for substitution in equation (2); for, with a sufficient number of values for  $x, y$  and  $P$ , we can solve this equation for the unknowns  $X, Y$  and  $v_i$ , provided we assume that  $v_i$  does not change with temperature. We know that  $X$  and  $Y$  are the same for each temperature, since a uniform sample of tissue was used throughout any one

<sup>1</sup> *Valonia* and *Nitella*, from which the cell-sap can be extracted and analysed, allow of a more accurate estimate of the internal concentrations; and it was found in the investigations of Osterhout [1926] on the uptake of hydrogen sulphide and of Osterhout and Dorcas [1927] on the uptake of carbon dioxide by *Valonia*, that the ratio  $[H^+ \text{ inside}]/[H^+ \text{ outside}]$  varied in the same manner as the ratio  $[HS^- \text{ outside}]/[HS^- \text{ inside}]$  or  $[HCO_3^- \text{ outside}]/[HCO_3^- \text{ inside}]$ . The deviations of these ratios from equality were rather great, but according to the writers were "such as might result largely from experimental error." It should be noted with reference to our subsequent remarks that in this case we are dealing with concentration of ions in the cell-sap and not with the average concentration of the ions in the cell as a whole.

<sup>2</sup> The results of this study could be explained qualitatively on the basis of the Donnan equilibrium in that, while the cation was absorbed in greater amount than the anion, rise of temperature, which caused the cation absorption to decrease, caused the anion intake concomitantly to increase. The opposite drift of cation and anion intake would result in the product being more nearly constant than if the drift had been in one ion alone. Constancy of external product, however, is not implicit in the application of the Donnan equilibrium as was suggested in the paper. Conditions may be such, and in the experiments were such, as to make the drift in the external product inappreciable.

experiment. If  $X$ ,  $Y$  and  $v_i$  determined in this way have values which are rendered improbable by other evidence, we shall have some ground for believing that the system is not of the type for which Donnan's equation holds.

Table I. *Equilibria in the absorption of 0.1 M ammonium chloride by carrot tissue at various temperatures*<sup>1</sup>.

Temp. ° C.	NH <sub>4</sub> <sup>+</sup>			Cl <sup>-</sup>			Ext. ionic product	Int. ionic product
	Final ext. conc. $(x_e) \times 10^3 M$	Final int. conc. $(x_i) \times 10^3 M$	Absorp- tion ratio $(\frac{xv_e}{x_e v_i})$	Final ext. conc. $(y_e) \times 10^3 M$	Final int. conc. $(y_i) \times 10^3 M$	Absorp- tion ratio $(\frac{y v_e}{y_e v_i})$		
4	-0805	·2143	2.662	·0930	·07676	0.8252	·00748	·01044
15	·0825	·1923	2.331	·0916	·09232	1.008	·00756	·01755
23	·0855	·1593	1.863	·0802	·1187	1.330	·00758	·01891

Let us consider for example the results obtained for the intake of ammonium chloride (Table I). It is perhaps reasonable to suppose tentatively that the tissue originally contained no appreciable amount of NH<sub>4</sub><sup>+</sup>, so that we may assume  $X = 0$ ; chlorine, however, is definitely known to be present in considerable quantity in some samples of carrot tissue [Pearsall and Ewing, 1924], and some may probably still have been left in the present instance in spite of the washing to which the tissue was subjected prior to the experiment. For  $P$ ,  $x$  and  $y$  we obtain the following values from the experimental results:

$P$	$x$	$y$
$7.48 \times 10^{-3}$	$1.95 \times 10^{-3}$	$0.70 \times 10^{-3}$
$7.55 \times 10^{-3}$	$1.75 \times 10^{-3}$	$0.84 \times 10^{-3}$
$7.58 \times 10^{-3}$	$1.45 \times 10^{-3}$	$0.08 \times 10^{-3}$

We may substitute these values in equation (2) and obtain the most probable values of  $Y$  and  $v_i$  by the method of least squares; by this procedure we find that

$$Y = 0.417 \times 10^{-3} \text{ g.-mols.,}$$

and

$$v_i = 17 \text{ cc.}$$

Let us also determine the values of the unknowns from the results recorded on the intake of potassium chloride. In this case we have to consider that potassium as well as chlorine is previously existent in the cell<sup>2</sup>, so that  $X$  will have a finite value. From the experimental results (Table II) we obtain the following values:

$P$	$x$	$y$
$8.72 \times 10^{-3}$	$1.04 \times 10^{-3}$	$0.27 \times 10^{-3}$
$8.76 \times 10^{-3}$	$0.96 \times 10^{-3}$	$0.31 \times 10^{-3}$
$8.68 \times 10^{-3}$	$0.94 \times 10^{-3}$	$0.42 \times 10^{-3}$
$8.69 \times 10^{-3}$	$0.88 \times 10^{-3}$	$0.47 \times 10^{-3}$

<sup>1</sup> Tables I-IV are based on the results of Petrie [1927], Tables V and VI on those of Stiles [1924]. The volume of solution employed in each case was 100 cc. The concentrations are expressed in gram-molecules per litre. The value of  $v_i$  is 9.1 cc. in Tables I-IV and 11.3 cc. in Tables V and VI.

<sup>2</sup> Stiles [1924] found that K<sup>+</sup> along with Ca<sup>++</sup> and Mg<sup>++</sup> diffused out from carrot tissue to replace the excess of Na<sup>+</sup> absorbed from a solution of NaCl. The previous existence of potassium in the tissue would be a possible interpretation of the small slope of the cation and anion curves for KCl absorption compared with those for NH<sub>4</sub>Cl (cf. Petrie [1927], Figs. 2 and 5).

Table II. *Equilibria in the absorption of 0.1 M potassium chloride by carrot tissue at various temperatures.*

Temp. °C.	K <sup>+</sup>			Cl <sup>-</sup>			Ext. ionic product	Int. ionic product
	Final ext. conc. $\left(\frac{x_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{x_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{x v_e}{x_e v_i}\right)$	Final ext. conc. $\left(\frac{y_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{y_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{y v_e}{y_e v_i}\right)$		
4	·0896	·1143	1.276	·0973	·02968	·3050	·00872	·00339
10	·0904	·1055	1.167	·0969	·03407	·3517	·00876	·00359
21	·0906	·1033	1.140	·0958	·04615	·4817	·00868	·00477
26	·0912	·0967	1.060	·0953	·05165	·5420	·00869	·00500

The number of equations is not sufficient in this case to permit the use of the method of least squares. We may, however, solve for the three unknowns by taking the equations in all possible combinations of three, the average of the results so obtained being as follows:

$$X = - \cdot 9523 \times 10^{-3} \text{ g.-mols.};$$

$$Y = - \cdot 3754 \times 10^{-3} \text{ g.-mols.};$$

and  $v_i = + \cdot 7243 \text{ cc.}$

Another series of values might be obtained by taking the alternative roots of  $X$  and  $Y$ ; they have been neglected, however, since these roots are considerably greater negative quantities than the above values.

Some results of Stiles [1924] also provide data which can be substituted in equation (2). These results were obtained by a similar technique, again using carrot tissue, only in this case the experiments were carried out at the same temperature with different external concentrations. Since the tissue remained turgid throughout the experiments, we may perhaps assume that the volume of the internal solute did not vary appreciably with the external concentration.

Taking for example the experiments with sodium chloride, we have the following data (Table V, p. 1076):

$P$	$x$	$y$
$6.860 \times 10^{-3}$	$2.52 \times 10^{-3}$	$0.83 \times 10^{-3}$
$5.380 \times 10^{-3}$	$3.61 \times 10^{-4}$	$1.58 \times 10^{-4}$
$4.778 \times 10^{-7}$	$3.81 \times 10^{-5}$	$2.58 \times 10^{-5}$

Substituting these values in equation (2) and solving simultaneously the three equations so obtained, we find that

$$X = - \cdot 3354 \times 10^{-3} \quad \text{or} \quad - \cdot 5758 \times 10^{-3} \text{ g.-mols.};$$

$$Y = + \cdot 007979 \times 10^{-3} \quad \text{or} \quad + \cdot 1076 \times 10^{-3} \text{ g.-mols.};$$

and  $v_i = 16.33 \quad \text{or} \quad 16.29 \text{ cc.}$

Now the volume of the whole tissue employed was, in the case of the ammonium chloride experiment, only 9.1 cc., and in the case of the sodium chloride experiment only 11.3 cc.; so that it is evident that the value of  $v_i$  is in these cases impossibly high. It is also unreasonable that either  $X$  or  $Y$  should be negative as is indicated in the cases of potassium chloride and sodium chloride. We are therefore led to the conclusion that the Donnan equation cannot be applied to these experiments.

The same conclusion can be reached from another aspect. We may take  $x$  and  $y$  of equation (2) as being the minimum values of  $x_i$  and  $y_i$  in equation (1), since they do not include any amount of the ions which may have been in the tissue at the commencement of the experiment; we may also take 9.1 or 11.3, as the case may be, and which we may express as  $v_i$ , as the maximum value of  $v_i$ , since the actual volume of the solute concerned is undoubtedly less than that of the whole tissue. The value of  $xy/v_i^2$  is thus a minimum for the inside product  $(X + x)(Y + y)/v_i^2$ . Now, if the Donnan equation applies, the outside product should equal the inside product, and hence the quantity  $xy/v_i^2$  should be less than the outside product, the more so the greater the value of  $X$ . In the results for potassium chloride (Table II), where one presumes that  $X$  is really comparatively high, the relationship actually holds; but in the case of ammonium chloride, where  $X$  is probably considerably less, instead of the

Table III. *Equilibria in the absorption of 0.1 M ammonium sulphate by carrot tissue at various temperatures.*

Temp. °C.	NH <sub>4</sub> <sup>+</sup>			SO <sub>4</sub> <sup>''</sup>			Ext. ionic product $\left(\frac{x_e y_e}{v_e^2}\right) \times 10^6$	Int. ionic product $\left(\frac{x_i y_i}{v_i^2}\right) \times 10^6$
	Final ext. conc. $\left(\frac{x_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{x_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{x_e v_e}{x_i v_i}\right)$	Final ext. conc. $\left(\frac{y_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{y_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{y_e v_e}{y_i v_i}\right)$		
5	·158	·4615	2.920	·0985	·0165	0.1674	·00246	·003515
12	·167	·3626	2.172	·0985	·0165	0.1674	·00275	·002169
17	·170	·2619	1.540	·0953	·0516	0.5420	·00275	·003538
21.5	·192	·0879	0.458	·0897	·1132	1.262	·00331	·000875

Table IV. *Equilibria in the absorption of 0.1 M calcium nitrate by carrot tissue at various temperatures.*

Temp. °C.	Ca <sup>++</sup>			NO <sub>3</sub> <sup>'</sup>			Ext. ionic product $\left(\frac{x_e y_e}{v_e^2}\right) \times 10^6$	Int. ionic product $\left(\frac{x_i y_i}{v_i^2}\right) \times 10^6$
	Final ext. conc. $\left(\frac{x_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{x_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{x_e v_e}{x_i v_i}\right)$	Final ext. conc. $\left(\frac{y_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{y_i}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{y_e v_e}{y_i v_i}\right)$		
4	·0917	·09122	·9947	·199	·01099	·0522	·003633	·0001102
8	·0930	·07693	·8271	·197	·03297	·1673	·003610	·0008362
14	·0950	·05495	·5785	·195	·05495	·2818	·003612	·001660
18	·0959	·04506	·4699	·185	·16480	·8910	·003283	·01225

outside product still exceeding  $xy/v_i^2$  although to a smaller extent, it is actually less. The same phenomenon tends to appear in the results for ammonium sulphate (Table III); and the external product is also considerably less than  $xy/v_i^2$  in Stiles's experiments with sodium and ammonium chlorides (Tables V and VI, p. 1076). Perhaps the same might hold for the experiments on calcium (Table IV) and potassium salts if the probably large values of  $X$  and  $Y$  were respectively added to  $x$  and  $y$ .

To make the internal product equal to the external product, either the value of  $v_i$  has to be greater than the volume of the tissue ( $v_i$ ), as resulted in



the calculations for ammonium and sodium chlorides; or the observed values of  $x$  and  $y$  have to be reduced, as resulted in the calculations for potassium and sodium chlorides, where  $X$  and  $Y$  in the one case and  $X$  in the other case became negative. There thus seems no doubt that the law of equality of products does not hold between the tissue and the external solution: in other words, the tissue as a whole cannot be treated as a simple Donnan system.

Table V. *Equilibria in the absorption of sodium chloride by carrot tissue at 20° (circa) in various concentrations.*

Init. ext. conc. $M$	Na <sup>+</sup>			Cl <sup>-</sup>			Ext. ionic product $\left(\frac{x_e y_e}{v_e^2}\right) \times 10^6$	Int. ionic product $\left(\frac{xy}{v_i^2}\right) \times 10^6$
	Final ext. conc. $\left(\frac{x_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{x}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{xy_e}{x_e v_i}\right)$	Final ext. conc. $\left(\frac{y_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{y}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{y y_e}{y_e v_i}\right)$		
0.1	·0748	·223	2.98	·0917	·07345	0.801	$·6860 \times 10^{-2}$	$·1638 \times 10^{-1}$
0.01	·00639	·03195	5.00	·00842	·0140	1.66	$·5380 \times 10^{-4}$	$·4474 \times 10^{-3}$
0.001	·000619	·00337	5.45	·000772	·00202	2.62	$·4778 \times 10^{-6}$	$·6808 \times 10^{-5}$

Table VI. *Equilibria in the absorption of ammonium chloride by carrot tissue at 20° (circa) in various concentrations.*

Init. ext. conc. $M$	NH <sub>4</sub> <sup>+</sup>			Cl <sup>-</sup>			Ext. ionic product $\left(\frac{x_e v_e}{v_e^2}\right) \times 10^6$	Int. ionic product $\left(\frac{xy}{v_i^2}\right) \times 10^6$
	Final ext. conc. $\left(\frac{x_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{x}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{xy_e}{x_e v_i}\right)$	Final ext. conc. $\left(\frac{y_e}{v_e}\right) \times 10^3 M$	Final int. conc. $\left(\frac{y}{v_i}\right) \times 10^3 M$	Absorp- tion ratio $\left(\frac{y y_e}{y_e v_i}\right)$		
0.1	·0871	·1141	1.31	·0941	·0520	0.553	$·8196 \times 10^{-2}$	$·5933 \times 10^{-2}$
0.01	·00428	·0506	11.8	·00678	·0285	4.20	$·2901 \times 10^{-4}$	$·1442 \times 10^{-2}$
0.001	·0000293	·00859	293.2	·0001923	·00715	37.2	$·5635 \times 10^{-8}$	$·6142 \times 10^{-4}$

#### THE POLYPHASE SYSTEM OF THE PLANT CELL.

There are various ways in which the tissue may differ from a simple Donnan system so as to explain these experimental results which we have been considering. The Donnan equilibrium applies to free ions only, whereas it is possible that some of the ions in the cell are bound as undissociated salts of protein or other substances, or some of the ions may be present in a state of physical adsorption and so be removed from solution. These ions would thus be present in the cell in addition to those which had entered to satisfy the product equation, and so would result in the internal concentration product being greater than that of the external concentrations. There is evidence that unless in considerable dilution the ionisation of protein salts is incomplete [Manabe and Matula, 1913; Hitchcock, 1923]; but to accept this hypothesis we should require to know the degree of ionisation of the particular salts concerned at their existing concentrations.

Another explanation of the inequality of the products might lie in the possibility, suggested by Butkewitsch and Butkewitsch [1925], that the degree

of permeability of the plasma-membrane is not the same in both directions: if inorganic ions could enter the cell with greater velocity than they could emerge, they would accumulate in greater amount in the cell than the simple Donnan theory would require. While, however, these properties have been shown to characterise some membranes [Wertheimer, 1923, 1924], we have not the same type of evidence that this is so for the material with which we are concerned in this discussion.

Whether either of these factors is responsible for the inequality of the concentration products cannot be determined at present; but, apart from any such possibilities, there are certain characters of the system involved which must tend to result in a higher value for the quantity  $(X + x)(Y + y)/v_i^2$  than for the product of the external concentrations. The plant cell is not a simple system, composed of a mere membrane enclosing a homogeneous solution, one of the ions of which is indiffusible: there are at least three phases between which the ions may be distributed—the external medium, the cytoplasm, and the vacuoles. Within the cytoplasm also, and perhaps in the cell-sap, there are micelles, or gel-particles, which can constitute yet other phases. Loeb [1924] has suggested in his work on the viscosity of gelatin and caseinogen solutions that between such particles and the continuous phase a Donnan equilibrium may become established<sup>1</sup>. Hence there are a number of phases, all of which are in equilibrium with one another; and it can be shown that the apparent internal ionic product, resulting from the collective effect of all the phases within the tissue under these conditions, must have a higher value than that of the external medium.

Let us consider for example the case where there are two internal systems. Suppose  $a_1$ ,  $b_1$  and  $a_2$ ,  $b_2$  represent the amounts of any pair of oppositely charged diffusible ions in the two internal systems respectively; and let  $v_1$  and  $v_2$  be the respective amounts of solvent in which they are dissolved. Finally, let  $R$  denote the value of the product of the concentrations of the two ions in the external solution at equilibrium.

When equilibrium has established itself the product of the concentrations of the two ions must be the same in each system; hence

$$R = \frac{a_1 b_1}{v_1^2} = \frac{a_2 b_2}{v_2^2} = \frac{a_1 b_1 + a_2 b_2}{v_1^2 + v_2^2}.$$

Let  $R'$  represent the apparent internal ionic product based upon the combined effect of the two internal systems; *i.e.*

$$R' = \frac{(a_1 + a_2)(b_1 + b_2)}{(v_1 + v_2)^2}.$$

We have now to determine the conditions governing the following relationship:

$$R' \geq R,$$

<sup>1</sup> Most investigators assume that a particle of a colloid, large or small, can be treated as a homogeneous solution surrounded by a membrane impermeable to one of the contained ions; we are not concerned here with the validity of this assumption.

$$\text{or} \quad \frac{a_1 b_1 + a_2 b_2 + a_1 b_2 + a_2 b_1}{v_1^2 + v_2^2 + 2v_1 v_2} \approx \frac{a_1 b_1 + a_2 b_2}{v_1^2 + v_2^2},$$

$$\text{or} \quad \frac{a_1 b_2 + a_2 b_1}{2v_1 v_2} \approx \frac{a_1 b_1 + a_2 b_2}{v_1^2 + v_2^2},$$

$$\text{or} \quad \frac{a_1 b_2 + a_2 b_1}{2v_1 v_2} \approx \frac{a_1 b_1}{v_1^2}.$$

Since

$$\frac{a_1}{v_1} \cdot \frac{b_1}{v_1} = \frac{a_2}{v_2} \cdot \frac{b_2}{v_2},$$

or

$$\frac{a_1 v_2}{v_1 a_2} = \frac{b_2 v_1}{v_2 b_1},$$

the expression becomes

$$\frac{1}{2} \left( \frac{a_1^2 b_1 v_2}{v_1^3 a^2} + \frac{a_1 b_1^2 v_2}{v_1^3 b^2} \right) \approx \frac{a_1 b_1}{v_1^2},$$

or

$$\frac{1}{2} \cdot \frac{a_1 b_1}{v_2^2} \left( x + \frac{1}{x} \right) \approx \frac{a_1 b_1}{v_1^2},$$

where

$$x = \frac{a_1}{v_1} \cdot \frac{v_2}{a_2} = \frac{b_2}{v_2} \cdot \frac{v_1}{b_1};$$

whence the expression becomes

$$x + \frac{1}{x} \approx 2.$$

Now

$$x + \frac{1}{x} > 2,$$

except when  $x = 1$ , i.e. except when

$$\frac{a_2}{v_1} = \frac{a_1}{v_2};$$

that is, the apparent internal product,  $R'$ , for a system in which there are two internal phases, will be greater than the external product  $R$ , except in the one case where the concentration of each of the ions is the same for the two internal phases. A similar conclusion could be drawn for a system of a greater number of internal phases.

The possibility of the concentration of any ion being the same in all the phases of the internal system may be dismissed. For it must be observed that, in plant tissues such as the root of the carrot, the intercellular space system, which from the practical point of view is included with the tissue, represents an internal phase. It is to be expected that this system contains ions in the same concentrations as in the external solution, so that it constitutes a phase which differs from the others within the tissue<sup>1</sup>.

#### THE COMPLICATION OF A MIXED AMPHOLYTE SYSTEM.

If the indiffusible ion in each phase of the internal system has the same type of charge, then the concentration of any diffusible ion of opposite charge in each of these phases, and hence its average concentration in all the internal phases together, should be greater than the concentration of the same ion in

<sup>1</sup> It is evident that a solution of colloid in which some of the colloid is in micellar form, enclosed in a collodion bag, constitutes such a polyphase system as we have been considering. The application of the Donnan equation to such a system is thus unjustifiable if the amount of micellar colloid is appreciable.

the external solution; similarly it follows that this ratio for any diffusible ion of the same charge as the indiffusible ion should be less than unity. Thus, if the indiffusible ion is a negative one, as is generally concluded to be the case in living tissues, we should have

$$\frac{(X+x)v_e}{x_e v_i} > 1,$$

and

$$\frac{(Y+y)v_e}{y_e v_i} < 1;$$

where  $X$  and  $Y$  are the amounts of the cation and anion respectively initially present in the tissue,  $x$  and  $y$  are the respective amounts of the same ions absorbed from the external solution,  $x_e$  and  $y_e$  are the final amounts in the external solution, and  $v_i$  is the volume of the internal, and  $v_e$  that of the external solution. Actually, taking  $xv_e/x_e v_i$  and  $yv_e/y_e v_i$  respectively as minimal values of the two above expressions, where  $v_i$  is the volume of the tissue employed, we find in certain cases (as will be seen from the accompanying tables) that both ratios may be greater than unity; and, since  $v_i$  cannot be greater than  $v_e$  it is obvious that the excess over unity may in reality be greater than is apparent.

This anomaly might be accounted for by assuming that some of the anions are bound in the undissociated state; but another possible interpretation lies in the supposition that, while indiffusible anions occur in one of two internal phases, the other contains indiffusible cations. For example, let  $K$  be the amount of the indiffusible cation in one phase and  $A$  the amount of the indiffusible anion in the other. In the former phase let  $x_1$  and  $y_1$  represent the amounts of the cation and anion respectively of a diffusible salt; let  $x_2$  and  $y_2$  have the same significance in the latter phase; and let  $x_e$  and  $y_e$  represent the amounts of the same two ions in the external solution. Also let  $v_1$  be the volume of solvent in the first phase,  $v_2$  the volume of solvent in the second phase, and  $v_e$  the volume of the external solution. The system may hence be visualised as follows:

$$\begin{array}{c|c|c} K & x_1 & y_1 \\ \hline & v_1 & \end{array} \quad \begin{array}{c|c|c} A & x_2 & y_2 \\ \hline & v_2 & \end{array} \quad \begin{array}{c|c} x_e & y_e \\ \hline v_e \end{array}$$

In such a system the following relationships will hold:

$$\frac{y_1}{v_1} > \frac{y_e}{v_e}; \text{ and } \frac{x_2}{v_2} > \frac{x_e}{v_e}.$$

With certain values of  $K$  and  $A$ ,  $y_1/v_1$  may be so much greater than  $y_e/v_e$  that the addition of  $y_2$  to the numerator and of  $v_2$  to the denominator still leaves

$$\frac{y_1+y_2}{v_1+v_2} > \frac{y_e}{v_e},$$

while similarly at the same time

$$\frac{x_1+x_2}{v_1+v_2} > \frac{x_e}{v_e},$$

so that in the case of both ions the apparent internal concentration can be greater than the external.

To illustrate the possibility of this we may take the following numerical example, in which we have a system containing only one diffusible electrolyte:

$$K=9; \quad x_1=1; \quad y_1=10 \quad | \quad A=19.5; \quad x_2=20; \quad y_2=0.5 \quad | \quad x_e=31.6; \quad y_e=31.6$$

$$v_1=100 \quad | \quad v_2=100 \quad | \quad v_e=1000$$

Here the apparent internal concentration is greater than the external in the case of both cation and anion; that is, for the cation

$$\frac{21}{200} > \frac{31.6}{1000};$$

and, for the anion,

$$\frac{10.5}{200} > \frac{31.6}{1000}.$$

It must be noted that, for this to hold good,  $K$  and  $A$  must be in different phases; if they were both in the same phase then we should have

$$\frac{x_i y_i}{v_i} = \frac{x_c y_c}{v_c}.$$

Stearn [1927] has recently brought forward evidence from different sources for the belief that protoplasm contains a mixture of ampholytes of different isoelectric points: moreover, in the case of carrot tissue with which we are particularly concerned in this discussion, the work of Cohn, Gross and Johnson [1920] indicates that two proteins are present, one isoelectric at about  $p_H$  4 and the other in the region of  $p_H$  8; and, since the normal reaction of the cell-sap is about  $p_H$  6 [Pearsall and Ewing, 1924] one of these proteins must be dissociated as an acid and the other as a base. If these proteins occur in the cytoplasm as separate colloidal particles, they would behave as separate phases of the internal system; so that there is a possibility that the cells of this tissue contain such an ampholyte system as we have been considering. In terms of this system of two proteins dissociated, the one giving rise to cations and the other to anions<sup>1</sup>, and occurring in different phases of a poly-phase system, we have therefore a hypothesis whereby we may interpret some of the phenomena of the ionic equilibria of the living cell; but to what extent such a hypothesis is a true explanation of the facts it remains for further data to show.

## THE EFFECT OF TEMPERATURE ON THE UNEQUAL INTAKE OF IONS.

It is of interest now to consider how the effect of temperature on the unequal intake of the two ions of an electrolyte, observed by Petrie [1927], can be correlated with this new conception; for temperature may be expected to produce various changes in the system we have been considering.

<sup>1</sup> The effect of the protein existing as a negatively charged ion will be supplemented by indiffusible organic acids, particularly the amino-acids, and the feebly dissociated carbohydrates such as the pentosans. We must not forget also the phosphatides, which like the proteins are amphoteric: Feinschmidt [1912] found the isoelectric point of lecithin (which was, however, probably impure) to lie between  $p_H$  2 and  $p_H$  4; he found also that lecithin could combine with proteins to give a compound with an isoelectric point different from that of either of the constituents.

Thus, since the solubility of proteins increases with temperature, the disperse phase in the protoplasm may be reduced by the passing of some of the micellar proteins into crystalloidal solution; one of the phases in the heterogeneous internal system would in this way tend to pass into another. On the other hand, the effect of temperature might be regarded as increasing the swelling of the micelles of protein: Jordan-Lloyd and Pleass [1927] have shown that the swelling of gelatin rises up to about  $18^\circ$  and it is only after this that increased solubility conspicuously manifests itself. Increased swelling would lead to an increase rather than a decrease in the volume of the micellar protein phase.

Such changes as these, however, could not explain the observed decrease in cation intake and increase in anion intake associated with rising temperature. It is true that they would alter the internal product; but, if electrostatic neutrality is to be preserved, such alterations could be achieved only by equal transference of ions of both charges from one phase to another.

The decrease of the difference between cation and anion intake as the temperature rises necessitates a decrease of the difference between indiffusible anions and cations in the tissue. If the tissue consisted of a single phase with an indiffusible anion, then the effect of temperature would be explained if the acidic dissociation constant of the ampholyte in the cells was decreased with rise of temperature. If, however, the system consists of two phases, one with colloidal anion and the other with colloidal cation, then the possibilities are considerably increased. If the dissociation of both decreases as the temperature is increased, the conditions of the whole system may be such that the dominating effect of the anion also decreases. For example, if the two internal phases were of equal volume, but the anion in one greater than the cation in the other, then halving their concentrations would still leave the anion dominant but to a decreased extent. The effect of temperature on ion uptake could, however, be reconciled with an increase of both indiffusible anions and cations in the tissue, the increase of the latter more than counterbalancing the increase of the former, and the more so the higher the temperature. The fraction of the one ampholyte which is in the form of anions will be  $\frac{k_a}{[H^+] + k_a}$ , where  $k_a$  is the acidic dissociation constant. Similarly, the fraction of the other ampholyte in the form of cations will be

$$\frac{k_b}{[H^+] + k_b},$$

where  $k_b$  is the basic dissociation constant and  $k_w$  is the dissociation constant of water. If  $k_a$  and  $k_b$  are increased by rising temperature the absolute increase in the fraction dissociated will be at a maximum in the case of the acidic dissociation when  $[H^+] = k_a$ , and in the case of the basic dissociation when  $[H^+] = k_w/k_b$ . Hence, if the hydrogen ion concentration is a long way removed from  $k_a$  and nearer to  $k_w/k_b$ , then the effect of the increase in cations may more than counterbalance the effect of the increase in anions. At present, however,

we have no knowledge of the dissociation constants of the substances in the system or of the way in which temperature affects them.

We do not suggest that our interpretation of the phenomena of ionic absorption by tissues in terms of the Donnan equilibrium is the true one; it does, however, seem possible that this equilibrium plays a part, even though the system is not a "simple" one as has been suggested in some papers.

#### SUMMARY.

The conception of a simple Donnan membrane equilibrium operating between two homogeneous phases is inadequate to explain the phenomena of ionic intake by plants. Experimental evidence indicates that the product of the apparent internal concentrations of cation and anion, estimated on the basis of the total volume of the tissue, may be greater than that of the concentrations of the two ions in the external medium, instead of equal to it, as the above conception would require.

It is pointed out that the interior of the tissue comprises a number of phases each of which may be in Donnan equilibrium with the external solution. The product of the apparent internal ionic concentrations resulting from the total effect of all these internal phases is shown under such circumstances to be greater than the external product.

Even in such a system as this, however, if the ratio of the apparent internal to the external concentration of a cation is greater than unity, the same ratio of the corresponding anion should be less than unity; whereas the experimental evidence indicates that both may at times be greater than unity. It is shown that this may be accounted for if we suppose that one internal phase contains indiffusible cations and that another contains indiffusible anions.

The effect of temperature on the relative uptake of the cation and anion of a salt can be interpreted in terms of its possible effect on the dissociation of the tissue colloids.

#### REFERENCES.

- Butkewitsch and Butkewitsch (1925). *Biochem. Z.* **161**, 468.  
 Cohn, Gross and Johnson (1920). *J. Gen. Physiol.* **2**, 145.  
 Feinschmidt (1912). *Biochem. Z.* **38**, 244.  
 Hitchcock (1923). *J. Gen. Physiol.* **5**, 35, 383, 397.  
 Jordan-Lloyd and Pleass (1927). *Biochem. J.* **21**, 1352.  
 Loeb (1924). *Proteins and the theory of colloidal behaviour* (New York).  
 Manabe and Matula (1913). *Biochem. Z.* **52**, 369.  
 Osterhout (1926). *J. Gen. Physiol.* **8**, 131.  
 Osterhout and Dorcas (1927). *J. Gen. Physiol.* **9**, 255.  
 Pearsall and Ewing (1924). *New Phyt.* **23**, 193.  
 Petrie (1927). *Austral. J. Exp. Biol.* **4**, 169.  
 Stearn (1927). *J. Gen. Physiol.* **10**, 313, 325, 369, 379.  
 Stiles (1924). *Ann. Bot.* **38**, 617.  
 Wertheimer (1923). *Pflüger's Arch.* **199**, 383; **200**, 82, 354; **201**, 488.  
 — (1924). *Pflüger's Arch.* **203**, 542; **206**, 162.

# CXXXIII. THE ISOLATION OF PURE *l*-PROLINE.

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*(Received July 5th, 1928.)*

DURING the last few years, investigations have been carried out in this laboratory with the object of discovering a method for a more quantitative and simple separation of the hydrolysis products of the proteins than those at present in vogue.

In the course of this work a method was discovered, some 18 months ago, for the separation in almost quantitative yield of pure *l*-proline. The proline thus isolated was found to differ appreciably in its properties from those ascribed to it in the literature. It was found, for example, to have a considerably higher laevorotation ( $[\alpha]_D^{18^\circ} = -86.7^\circ$ ) compared with that previously mentioned in the literature ( $[\alpha]_D^{18^\circ} = -80^\circ$ ), and was found furthermore to crystallise from water, and to be only slightly soluble in cold absolute alcohol, although it is more readily soluble in the hot solvent. Recrystallisation from hot alcohol forms, in fact, a very convenient method for its purification.

It was intended to withhold the publication of these results until the general researches had reached a more advanced stage. In the meantime, however, attention has been called to a paper published a few months ago by Kapfhammer and Eck [1927] which has only just come to the notice of the author. As the results obtained by the author confirm, in some measure, those of Kapfhammer and Eck (except that the rotation has been found to be some  $2^\circ$  higher than mentioned by them), and furthermore as the method employed appears to be simple and entails the use only of the most common laboratory reagents, it has been thought advisable to publish it at the present stage, withholding for the present the details of the separation of the other hydrolysis products.

Kapfhammer and Eck employed for the separation a reagent known as Reinecke's acid,  $\text{H}[(\text{SCN})_4\text{Cr}(\text{NH}_3)_2]$ , the ammonium salt of which is prepared by fusing together ammonium thiocyanate and ammonium dichromate. This reagent has been found by them to be a specific precipitant for proline and hydroxyproline.

The method employed by the author depends, in the first instance, on the separation of the hydrolysis products into three fractions by means of their copper salts, which have differing properties.



- I. Copper salts soluble in water and in methyl alcohol.
- II. Copper salts soluble in water, but insoluble in methyl alcohol.
- III. Copper salts insoluble in both water and methyl alcohol.

The success of the method depends on the employment of a special technique which is described in detail below. The proline is found in the first fraction, together with, in the case of gliadin (which was the protein first employed in the evolution of the method), valine, hydroxyvaline, and the peptide phenyl-alanylproline, first isolated by Osborne and Clapp [1907]. These are insoluble in absolute alcohol, whereas proline is soluble. From the alcohol-soluble portion, proline can be precipitated almost quantitatively as the picrate. There are also among the copper salts soluble in methyl alcohol (*a*) a product soluble in alcohol but giving no picrate and (*b*) a product giving a picrate soluble in ether; these fractions are at present being investigated.

The fraction giving copper salts soluble in water but not in methyl alcohol contains glutamic acid, the bases, glycine, alanine and serine.

The fraction giving copper salts insoluble in water and in methyl alcohol contains phenylalanine, leucine and aspartic acid. Methods are in use, or are being worked out in this laboratory, for the separation of the constituents of these fractions, and will be published in due course. Pure proline has also been prepared in this laboratory, by the method here described, from glutenin by Mr E. L. Hill, from gelatin by Mr Hand, and by other workers from other sources.

#### EXPERIMENTAL.

500 g. of dry wheat gliadin were hydrolysed by slowly adding the protein to four times its bulk of hot 25 % sulphuric acid, and boiling for 24 hours under a reflux condenser. The hydrolysate was now cooled, diluted to 4 litres, and the humin material filtered off and washed. To the filtrate and washings was added hot saturated baryta solution roughly equivalent to the sulphuric acid present. The precipitated barium sulphate was filtered off and washed seven times by boiling with water. The filtrate and the washings from the barium sulphate were now concentrated to about 2 litres, and barium and sulphuric acid quantitatively removed.

The solution of amino-acids was next heated in a large porcelain evaporating basin, on a water-bath, and copper carbonate was added, little by little, until there was no more effervescence. A fair quantity of copper carbonate was now added, and the solution concentrated to a thick syrup. The mass was diluted with water, and the excess of copper carbonate, together with any insoluble copper salts, was filtered off and thoroughly washed with water. The filtrate was once again evaporated to a thick syrup, with the further addition of copper carbonate. When quite viscous, the material was treated with dry acetone, and after standing for a few minutes with this liquid the acetone was poured off and a fresh quantity added. After five or six treatments with acetone the copper salts granulated rapidly. The acetone was renewed several times after

this, and finally the copper salts were lightly ground in a mortar, when they fell rapidly to a fine powder. The acetone was removed as far as possible by filtering through a Büchner funnel, and the copper salts further dried by leaving overnight in a vacuum desiccator, over sulphuric acid. The solid was finally dried in an electric oven at  $110^{\circ}$  for a short time. The whole success of the method depends on the rapid and efficient drying which the acetone effects; no other method has been found for completely drying the copper salts, which is essential to the success of the separation.

The dried copper salts were now extracted by shaking mechanically in a stoppered bottle with twice the bulk of dry methyl alcohol. After shaking for 2 hours, the alcohol was filtered off and the residue shaken once again with methyl alcohol for 1 hour. This extraction was repeated six times, by which time only negligible quantities of material were being dissolved out.

This methyl alcohol-soluble portion of the copper salts was freed from the solvent by distillation, again granulated with acetone and finally dried for an hour at  $110^{\circ}$  as before. It was then re-extracted with methyl alcohol, when the whole material dissolved. On dilution, and standing overnight, however, a quantity of copper salt gradually settled out. This, when dry, weighed only 10 g.

The alcohol was again distilled off and the syrupy copper salts were taken up with water and freed from copper by means of hydrogen sulphide. The free amino-acids, after careful washing of the copper sulphide precipitate, were concentrated to a syrup and treated with absolute alcohol. A large quantity of material was precipitated; this was filtered off and washed, and the filtrate again evaporated down and taken up with absolute alcohol. When a fraction was obtained which was quite soluble in absolute alcohol, the watery solution of the same was treated with picric acid in sufficient quantity to combine with the nonamino-nitrogen present, and the solution boiled. On cooling, a somewhat soft and oily precipitate was obtained, which on filtration and extraction once with ether yielded a crystalline mass of proline picrate. This was recrystallised once from water and decomposed by acidifying with sulphuric acid, extracting the picric acid with ether and evaporating the solution to dryness after removal of the sulphuric acid, when pure proline was obtained. Large quantities of proline have been obtained by the author using this method, several hundred grams of the picrate having been prepared from gliadin alone.

*Properties.* Pure proline is a white, non-deliquescent solid. It crystallises quite easily from strong aqueous solutions in the form of long needles. It is not very soluble in cold absolute alcohol, but dissolves readily in the hot solvent, crystallising out on cooling, also in needle-shaped crystals. It may also be recrystallised from *iso*-propyl alcohol. It melts with decomposition at  $215^{\circ}$ . It gives absolutely no amino-nitrogen in the van Slyke apparatus.

*Analysis.* N (Kjeldahl)—Found: 12.20 %. Calculated: 12.17 %. C and H—2.370 mg. gave 4.540 mg.  $\text{CO}_2$ , 1.682 mg.  $\text{H}_2\text{O}$ . Found: C, 52.25 %;

H, 7.90 %. Calculated: C, 52.14 %; H, 7.88 %. Rotation: (i) 0.6206 g. in 50 cc. water gave, in a 2 dm. tube, a rotation of  $-2.15^\circ$ ; whence  $[\alpha]_D^{18} = -86.6^\circ$ . (ii) 1.734 g. in 50 cc. water gave, in a 2 dm. tube, a rotation of  $-6.02^\circ$ ; whence  $[\alpha]_D^{18} = -86.8^\circ$ .

*Derivatives.* The picrate may be prepared by adding the requisite quantity of picric acid to the hot aqueous solution of proline and cooling; the picrate is thus readily obtained pure. It is practically insoluble in cold water, although it has a large temperature-coefficient of solubility. Attempts to prepare the picrate by the method of Alexandroff [1905] were not so successful. The picrate thus prepared had m.p.  $148^\circ$ . The m.p. quoted in the literature is  $152-4^\circ$ ; only once has a picrate of this m.p. been obtained. A mixed m.p. of this sample with that of m.p.  $148^\circ$  gave  $153-4^\circ$ . The picrate melting at  $148^\circ$  crystallised in long golden-yellow needles, while that melting at  $154^\circ$  was in the form of short, dull brown needles. Both picrates on decomposition gave a proline with the same optical rotation. Picrates of proline prepared by other workers in this laboratory have been found invariably to melt at  $148^\circ$ .

The phenylhydantoin is formed quantitatively by shaking proline with phenyl isocyanate in molecular proportions, keeping alkaline with caustic soda; the solution is then acidified with hydrochloric acid, and the strongly acid solution boiled for 5 minutes. On cooling, the phenylhydantoin crystallises out; m.p.  $143-4^\circ$ . If *l*-proline and phenyl isocyanate are warmed together for a few minutes and the resultant product crystallised from alcohol, pure *dl*-prolinephenylhydantoin is formed in good yield; m.p.  $118^\circ$ .

#### SUMMARY.

A method is given for the preparation of pure *l*-proline, which has a distinctly higher rotation than that stated in the literature. The method depends on a technique for separating the copper salts of protein hydrolysis products into three fractions. From the fraction which is soluble in methyl alcohol the proline can be prepared by isolation in the form of a picrate. The yield appears to be nearly quantitative. The pure compound is only slightly soluble in cold alcohol, though readily soluble in the hot solvent, from which it may be easily recrystallised.

The best thanks of the author are due to Professor S. B. Schryver, F.R.S., under whose direction the work was carried out. The research was undertaken under the auspices of the Adhesives Committee of the Department of Scientific and Industrial Research.

#### REFERENCES.

- Alexandroff (1905). *Z. physiol. Chem.* **46**, 17.  
Kaphammer and Eck (1927). *Z. physiol. Chem.* **170**, 294.  
Osborne and Clapp (1907). *Amer. J. Physiol.* **18**, 123.

## CXXXIV. THE FAT, LIPIN AND CHOLESTEROL CONSTITUENTS OF ADRENALS AND GONADS IN CASES OF MENTAL DISEASE.

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*(Received June 28th, 1928.)*

THE late Sir Frederick Mott, who suggested and initiated this work, stated that in his opinion, the fat, cholesterol and lipin substances of the nervous system and of the gonads were possibly manufactured in the cortex of the suprarenal gland, from which the material was carried by the blood for the nutrition of the gonad and developing brain. The present work was therefore undertaken upon his suggestion with the object of showing the possible relations between the suprarenal and gonad fatty constituents and their relative proportions in cases in which mental disorder is a marked feature. There are often marked histological changes in the gonads in cases of mental disorder and therefore such balance as possibly exists in the normal is most likely to be disturbed in these cases.

The adrenal cortex is essential to life in the higher animals. The experiments of Biedl [1910] show that the lipin-containing cortex is even of more vital importance than the medulla. Chauffard, Laroche and Grigaut [1920] found that the cholesterol content of the normal gland was as much as 5.5 % of the fresh tissue, *i.e.* twice that of any other organ. The cortex cells of the healthy adrenal are almost filled with fatty constituents of which cholesterol and doubly refracting lipins form the greater proportion. The disappearance of a large amount of these constituents in acute infection, muscular exhaustion and many chronic diseases, suggests that it is the deficiency consequent upon the exhaustion of these vital materials which causes death, and the condition is probably parallel to that found in animals after adrenalectomy.

There is little agreement, however, among various investigators regarding either the function of the adrenal in fat metabolism, or its relationship to the sex glands and brain tissue.

Mott and his collaborators [1917, 1920, 1923], and other workers, have found that morbid conditions of the sex glands were often accompanied by deficiency or atrophy of the adrenal cortex. Developmentally the gonads and adrenals are associated, since both are formed in the embryo from similar and proximate mesoderm cells of the genital ridge. A physiological association probably continues throughout life.

In animals where sexual activity varies with the season of the year, there

is a corresponding increase in the size and lipin content of the adrenal parallel with the sex gland activity. It has been claimed by Lespinasse that precocious sexual development was experimentally produced in a cockerel by transplantation of adrenals. Somewhat analogous changes occur, simultaneously with changes in the ovary, in the cortex cells of the adrenal during the oestrous cycle, and enlargement of the cortex and increase in cholesterol take place during pregnancy (Grigaut).

Glynn suggests that the adrenal controls the development and differentiation of the sex cells. Elliott however asserts that the adrenal is chiefly associated with the muscular system. Chauffard [1918] has shown that the cholesterol content of the foetal adrenal increases greatly after the fifth month.

The adrenal of the human foetus is relatively large, due to a wide "boundary zone" [Elliott and Armour, 1911]. Although this zone is not particularly rich in lipin before birth, later its cells become so filled with doubly refracting fatty constituents that it is with difficulty distinguishable as a separate zone.

In the anencephalic foetus this particular area is absent and the gland resembles that of other foetal mammals, which further emphasises the possibility of a connection between the adrenal cortex and the developing brain.

The suggestion that the adrenal synthesises cholesterol was put forward by Chauffard but the experiments of Bauman and Holly [1923] and more recently of Randles and Knudson do not support this.

Analyses of the cholesterol and lipin content of the adrenal and sex glands have been carried out by a number of workers. The figures for cholesterol in the human adrenal obtained by Chauffard and other French investigators range from 0.2 % to 9.0 % for fresh tissue and illustrate the great variations which may occur in the amounts present; they suggest that this variation is chiefly dependent upon the cause of death, the lowest contents being found after septic conditions such as tuberculosis or septicaemia.

In the present investigation the following constituents were separately determined: lecithin (including kephalin), sphingomyelin, neutral fat, fatty acids, cholesterol ester and free cholesterol. The quantity of lecithin was checked by phosphorus estimation and the neutral fats were found both directly from the fatty acid and by difference from total extract.

#### METHODS.

*Preparation of the tissue.* The glands were collected as soon as possible after death, carefully dissected free from fat and fibrous tissue and weighed. Small portions were preserved for histological examination and the remainder was finely minced. The testis was freed from the epididymis and capsule. The cortex and medulla of the adrenal were not separately investigated. The minced glands were preserved and fixed in 30 cc. of freshly prepared 3 % metaphosphoric acid. This reagent coagulated the proteins thus rendering the fatty constituents more accessible to the solvent action of the extracting fluids. After 24 hours the aqueous fluid was removed and discarded, the tissue washed

with water and carefully dried with bibulous paper. 5 cc. per g. of a mixture of alcohol and ether (2 : 1) were then added; the tissues could be preserved indefinitely under these conditions. For estimation the solvent was decanted and evaporated, and the remaining tissue carefully dried by a current of warm air. The residue was ground to a fine powder and dried in a desiccator after which the remaining fatty material was extracted by the special methods given below.

*Extraction of the dried tissue.* Owing to the difficulty of complete extraction of the tissues, it was necessary to devise a simpler and more rapid method of extracting the fats. This was effected by four successive digestions with alcohol and ether in simply constructed bombs at 150°. A sketch of the bomb employed is given in Fig. 1, the length of the tube being 4·5 inches and the internal diameter 1 inch.

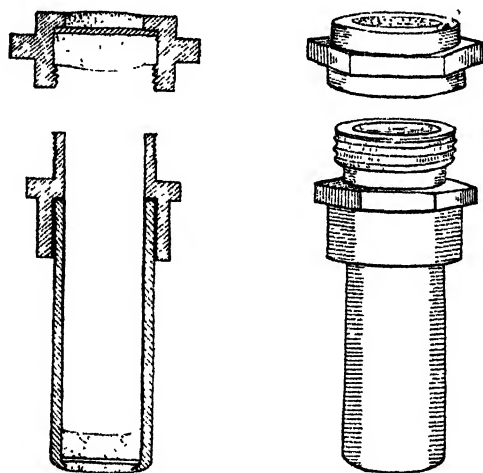


Fig. 1. Bomb for extraction of dry tissue.

The dotted, shaded portions indicate soft solder which forms an effective seal without the use of any washer whatsoever. Each bomb was tested in boiling water using ether.

The solutions obtained by this method of extraction were combined, evaporated and the fat added to that obtained by evaporating the solvent previously decanted from the tissues. The whole was then dissolved in a small volume of chloroform and the slight residue of non-fatty extractive discarded. In order to remove any phosphoric acid which might possibly be retained from the fixing fluid, about 0·1 g. of barium carbonate was added to the chloroform solution which was allowed to stand 2 hours with occasional rotation and then filtered; the residue was thoroughly washed with chloroform, and the washings were added to the extract. (The residue was decomposed with 20 % hydrochloric acid to liberate the fatty acid which was estimated separately.) The purified extract was again carefully evaporated, thoroughly dried, weighed, and dissolved in a fixed volume, usually 25 cc., of alcohol containing a small proportion of chloroform. Suitable quantities were then taken for investigation of the various constituents as follows.

## ESTIMATION OF THE CONSTITUENTS OF THE EXTRACT.

A. *Lipins containing phosphorus.*

10 cc. of the solution were evaporated and treated with 10 cc. pure acetone. The flask was occasionally rotated for 2 hours. The solution was then decanted for future examination and the operation repeated<sup>1</sup>. The acetone-insoluble phosphatides were next dissolved in a small quantity of ether and reprecipitated with acetone. This was decanted and mixed with the above soluble fraction. Three similar precipitations were necessary owing to the solubility of the lecithins in the fatty mixtures. The final residue consisted of mixed lipins containing phosphorus from which the sphingomyelin was separated by first extracting with hot alcohol and then allowing the mixture to cool, the sphingomyelin being precipitated. The precipitate was washed with ether and the phosphorus estimated as below. The remainder of the lipins containing phosphorus, consisting of lecithin and kephalin, was obtained by evaporating the alcohol. It was weighed and then the amount of phosphorus determined as below.

*Phosphorus estimation.* About 0.1 g. of the lipin was transferred to a small nickel crucible, and for each 0.05 g. of phosphatide 0.5 cc. of a saturated solution of magnesium nitrate and about 0.02 g. of magnesium oxide were added, the whole being allowed to evaporate slowly on a hot plate. The crucible was then heated with a Bunsen burner, at first cautiously, then strongly.

The ash was dissolved in 5 cc. of 50 % nitric acid and 15 cc. of 34 % ammonium nitrate were added, the solution heated to 100° and the phosphate precipitated with 10 cc. of 3 % ammonium molybdate. After standing for 12 hours the supernatant liquid was decanted through a filter, the phosphomolybdate washed with dilute nitric acid and dissolved in 10 cc. of 2 % ammonia. After transferring to a clean vessel, adding 0.5 cc. of molybdate solution and heating to 100°, 5 cc. of 10 % nitric acid were poured in slowly. The ammonium phosphomolybdate was reprecipitated, collected on a Gooch crucible, washed, dried and weighed. The factors by which the weights of the molybdate precipitate were multiplied were: for phosphorus, 0.0163; for lecithin (M.w. 770), 0.405; for sphingomyelin (M.w. 750), 0.396.

Sphingomyelin contains 43 % of lignoceric acid [Levene, 1916]; and the lecithin about 72 % of stearic, palmitic and linolic acids. These figures were used in calculating the lipin and fatty acids which appear as a portion of the total fatty acids estimated below.

<sup>1</sup> The solutions decanted were evaporated, the residue was dissolved in a small quantity of ether, acetone added and allowed to stand several hours or overnight. The precipitate, if any, was added to the phosphorus-containing lipins and the supernatant liquid reserved and used for the determination of cholesterol and such free fatty acid as was not removed by the barium carbonate.

B. *Cholesterol.*

1. *Free cholesterol* (modification of Windaus's [1910] method). 2 cc. of the lecithin-free extract (see note, p. 1090) were evaporated and the fats dissolved in 5 cc. of hot Caminade's [1922] acetone mixture (acetone 73, alcohol 9, water 18 parts).

A slight excess of digitonin in hot Caminade's solution was added and the precipitate allowed to stand for 2 hours at ordinary temperature. The digitonide precipitate was removed and dissolved in 5 cc. of boiling 90 % alcohol. After standing overnight at 18° it was collected in a Gooch crucible, washed with hot water and with a few drops of alcohol and ether, dried at 100° and weighed.

A correction of 0.0002 g. was added to the weight of the precipitate to allow for its solubility in the 5 cc. of alcohol. Factor for cholesterol 0.243.

- Notes.* (a) The precipitation in Caminade's solution is immediate and quantitative, but in presence of relatively large quantities of fat reprecipitation from alcohol is desirable.  
 (b) Washing with hot water and the prescribed volume of alcohol and ether does not dissolve a weighable quantity of digitonide [Mueller, 1917].  
 (c) If the quantity of cholesterol in the extract is very small a larger quantity of extract should be taken since a precipitate weighing 0.0100 g. or more is necessary for accurate determination.

2. *Total cholesterol.* 2 cc. of the extract were evaporated and dissolved in 5 cc. benzene; a quantity of sodium metal, rather more than the weight of total fat, and then 15 cc. of absolute alcohol were added.

The solution was heated under reflux for 8 hours, evaporated just to dryness and the cholesterol extracted repeatedly from the residue with warm light petroleum. The combined extracts were washed with water to remove traces of alkali, evaporated, the residue dissolved in 5 cc. 90 % alcohol, and the cholesterol determined as above. The difference between the total and free cholesterol is the ester cholesterol. The fatty acid was calculated by assuming 40 % to be present in the ester [cf. Mair, 1912].

C. *Total fatty acids.*

These were liberated by heating under reflux 2 cc. of the extract with 1 cc. of 50 % NaOH in 15 cc. alcohol for 4 hours. The alcohol was then removed, the soaps were dissolved in water and transferred to a separator, decomposed with slight excess of 20 %  $\text{H}_2\text{SO}_4$  and the free fatty acids extracted with light petroleum.

The extract was washed with water until the washings were neutral to methyl orange. The organic acids were then titrated by adding a known volume of  $N/10$  NaOH and titrating the excess with  $N/10$   $\text{H}_2\text{SO}_4$  from a micro-burette in presence of neutralised alcohol using phenolphthalein as indicator. The above method gave satisfactory results with experimental mixtures.

This process was necessary because it is comparatively difficult to hydrolyse kephalin [Levene and West, 1916].



*D. Free fatty acids.*

2 cc. of the lecithin-free extract were evaporated to dryness, dissolved in 5 cc. light petroleum, washed in a separator until the washings were neutral and then titrated as in C. The amount of fatty acids was calculated assuming a molecular weight of 280 in all cases. The fatty acids removed from the extract by the barium carbonate were added to those calculated from the above titration in order to find the quantity originally present in the extract.

RESULTS OF ANALYSIS OF THIRTY-EIGHT PAIRS OF GLANDS FROM MENTAL HOSPITAL PATIENTS.

The glands were obtained from autopsies at the Birmingham Mental Hospitals and include 29 male and 9 female cases. It has so far, however, been impracticable to obtain normal human specimens for control. One doubtfully normal specimen has been obtained from a case of veronal suicide and another from a case of accidental death, whose previous history cannot be obtained. For the purpose of comparison the cases were grouped according to age and psychiatric classification.

*Total extractives from the glands.*

Table I shows the average percentage of extractives in the fresh and dry tissues for the total number of glands.

Table II gives the human series arranged in age groupings.

Table I. *Total fatty extractives.*

Group	No. of cases	% in fresh tissues		% in dry tissues	
		Adrenal	Gonad	Adrenal	Gonad
Whole series	46	11.2	4.3	45.1	23.3
Human series	38	12.4	3.61	47.2	23.0
General paralysis	8	10.9	3.67	39.6	22.6
Dementia praecox	5	8.8	3.7	45.4	23.0
Confusional insanity	6	14.1	3.98	53.4	25.6
Senile dementia	5	19.4	3.6	63.9	28.0
Epileptic insanity	6	13.5	3.43	54.4	20.6
Normal (?) cases	2	12.5	2.86	48.6	23.6
Sheep	3	6.65	3.29	36.4	27.6
Guinea-pigs (mixed glands)	—	19.2	4.25	57.0	27.6
Sow	1	3.63	4.63	15.3	21.1
Cow	2	4.7	—	21.6	—

Table II. *Human cases in age groups.*

Group	No. of cases	% in fresh tissues		% in dry tissues	
		Adrenal	Gonad	Adrenal	Gonad
Under 20 years	4	7.16	2.92	45.7	21.9
20-40 years	11	12.4	3.58	46.2	23.8
40-60 years	16	13.8	3.67	48.5	24.4
Over 60 years	7	17.5	3.31	61.2	21.1

The figures for the analyses of the individual glands show that the gonads, whatsoever their source, contain approximately constant amounts of fat in both dry and fresh tissues; the fatty content of the adrenals however varies considerably.

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Amongst the human adrenals the lowest percentages were found in the dementia praecox group and the highest figures in senile dementia. The extractives of the dried adrenal tissue showed definite increase with the age of the subject; this was not due entirely to the glyceride fatty acids since the extractives contained a high proportion of phosphorus-containing lipins.

The figures for the extractives from the adrenals of the cow (21.6 %) and the sow (15.3 %) agree well with those given by Fenger [1916].

## *Chemical composition of the fatty extractives.*

The average percentages of the constituents of the fatty extractives are tabulated for the various groups in Tables III, III A, IV and V.

Table III. *Constituents of extractives %.*

Group	Lecithin	Sphingo- myelin	Total lipin	Free chole- sterol	Chole- sterol ester <sup>1</sup>	Total chole- sterol	Neutral fat	Free fatty acids
Adrenal series	32.9	4.43	37.3	3.72	15.9	13.1	35.9	5.6
Gonad series	37.1	4.48	41.6	7.0	9.55	10.5	35.7	4.2
Human adrenals	33.1	4.55	37.7	3.51	13.7	11.7	38.5	5.1
Human gonads	38.8	4.80	43.6	5.65	10.0	11.6	39.9	4.1
2 normal (?) human adrenals	26.3	0.6	37.6	5.4	21.7	18.5	39.7	5.5
2 normal (?) human testes	37.7	5.43	43.1	8.1	17.3	13.8	34.4	4.9

Table III A. *Constituents of extractives % (animals).*

Group	Lecithin	Sphingo- myelin	Total lipin	Free chole- sterol	Chole- sterol ester <sup>1</sup>	Total chole- sterol	Neutral fat	Free fatty acids
Sheep adrenals	52.6	6.0	58.6	10.8	6.52	14.8	19.3	5.6
Sheep testes	40.0	6.1	46.1	8.6	9.8	14.4	34.1	4.2
Sow adrenals	20.0	2.1	22.5	5.2	2.97	6.92	63.8	3.5
Sow ovary	9.15	1.79	10.9	3.75	21.9	16.9	50.5	3.6
Guinea-pig adrenals	52.7	4.4	57.1	1.56	14.25	10.1	22.9	3.15
Guinea-pig testes	70.4	4.4	74.8	1.85	12.59	9.4	6.5	4.1
Cow adrenals	34.0	5.7	39.7	5.85	0.01	5.9	47.8	6.6

Table IV. *Constituents of extracts % (chief mental groups).*

Mental groups	Lecithin		Sphingomyelin		Total phosphatide		Neutral fat	
	A	G	A	G	A	G	A	G
General paralysis	31.9	33.3	3.36	4.45	35.3	37.8	37.3	41.1
Dementia praecox	27.3	43.3	5.61	6.00	32.9	49.3	33.2	25.3
Epileptic insanity	43.5	34.8	3.83	4.08	47.3	38.9	22.3	43.4
Confusional „	14.3	27.2	3.74	4.1	18.0	31.3	59.3	55.0
Senile dementia	33.3	46.7	4.3	2.6	37.6	49.3	41.6	30.0

Mental groups	Free cholesterol		Cholesterol ester <sup>1</sup>		Total cholesterol	
	A	G	A	G	A	G
General paralysis	3.13	7.25	19.1	10.9	14.9	13.8
Dementia praecox	3.64	4.29	22.8	15.6	17.6	13.9
Epileptic insanity	3.36	7.99	20.7	7.6	16.0	12.4
Confusional „	1.62	2.42	15.3	6.6	10.7	6.4
Senile dementia	3.14	4.58	13.4	6.0	11.2	7.2

<sup>1</sup> Calculated assuming 40 % content of fatty acid [cf. Mair, 1912].

Table V. *Constituents of extracts % (age groups).*

Age group	Lecithin		Sphingomyelin		Total phosphatide		Neutral fat	
	A	G	A	G	A	G	A	G
Under 20 years	31.8	35.8	3.9	3.9	35.7	39.7	55.3	44.2
20-40 years	35.4	42.1	5.9	5.07	41.3	47.2	31.5	33.7
40-60 years	29.0	35.2	3.0	3.2	32.2	38.4	40.1	40.8
Over 60 years	41.3	38.6	3.9	3.9	45.2	42.5	37.0	45.3

Age group	Free cholesterol		Cholesterol ester		Total cholesterol	
	A	G	A	G	A	G
Under 20 years	3.35	3.96	13.3	8.83	11.3	9.3
20-40 years	4.3	5.41	16.3	10.4	16.3	11.2
40-60 years	3.14	5.62	18.6	9.4	14.6	11.3
Over 60 years	3.09	7.05	13.7	4.8	10.6	9.9

The group of confusional insanity shows low values of lecithin and cholesterol, especially free cholesterol. The epileptic group has high figures for the lipins containing phosphorus. The latter observation is opposed to the result obtained by Papea and Eustatziu [1925], who found, in two cases of death after prolonged epilepsy, that the suprarenals showed considerable fibrosis and disappearance of cortex lipins. Our analyses show an average cholesterol value in the adrenals of epileptics and in no individual case was the amount much less than the average for the total glands.

The average figure for phosphatide content of human testes was 43.6 % of the extract (1.58 % of the dry tissue). This is somewhat higher than that obtained by Mann in cases of mental disorder (half of which, however, had carcinoma).

One of the specimens, the testes of a senile dement (A. E. K.), contained a very high proportion of cholesterol ester (29 %). These glands weighed only 2.26 g. and were very fibrous with hard, thick capsules.

Guinea-pig adrenals were found to contain a very high proportion of phosphorus-containing lipins (57 %). Similar results are quoted by Elliott and Tuckett [1906].

One specimen of adrenal from a cow contained no cholesterol esters, although a moderate amount of free cholesterol was present. A similar absence of combined cholesterol has been reported by Sorg and Jaffé [1924]. The reverse condition, *i.e.* absence of free cholesterol and presence of ester cholesterol, was observed by Rosenheim and Tebb [1909] in the adrenals of cattle.

#### *Relative proportions of phosphatide and cholesterol.*

In the investigation of a possible relationship between the amounts of total phosphatide and total cholesterol (free cholesterol and cholesterol from the esters) an estimate was made of the relative proportion in which these constituents were present in each gland.

It was found that in half the adrenals the relative proportion of cholesterol to phosphatide was between 1 : 1.5 and 1 : 3.0, the remainder differing widely from these figures; in about one-third of the gonads the proportions were

between 1 : 2.0 and 1 : 5.0. The gonads showed a progressive decrease in the lipin-cholesterol proportion when considered in relation to the age grouping (Table VI).

It is interesting to note that in the group of confusional insanity the adrenals had a remarkably low proportion of phosphatide to cholesterol, while the lipin-cholesterol proportion in the gonads remained high. There is thus a wide variation in this class which exceeds any other grouping. The low lipin-cholesterol proportion in the adrenal was due to the low percentage of phosphatide and suggests that there is a relation between the disappearance of this constituent and that process which has been shown to occur in toxic conditions, existing over long periods, by Elliott and by Mott. These workers found that chronic infection results in marked diminution of cortex lipoids.

The main object for which this work was undertaken was to determine whether chemical evidence supported the view of the function of the adrenal in elaborating the cholesterol and phosphatide for the developing and functioning gonads. It is apparent that if these constituents are not changed or further elaborated the ratio obtained by dividing the proportions of phosphatide to cholesterol in the adrenal by the proportion in the gonads would be unity. These ratios have been accordingly calculated and are given in Table VI. It was found that many of the ratios varied widely from the

Table VI. *Relative proportions of cholesterol and phosphatide.*

Average for	Whole series	General paralysis	Dementia praecox	Epileptic insanity	Confus insanity	Senile dementia	Under 20 years	20-40 years	40-60 years	Over 60 years
Adrenal	2.8	2.4	1.95	3.0	1.7	3.4	3.1	2.5	2.2	4.3
Gonad	4.0	2.7	3.55	3.1	4.8	3.7	4.25	4.2	3.4	4.2
Ratio	0.70	0.89	0.55	0.97	0.35	0.92	0.73	0.60	0.65	1.0

expected figure and therefore this view is not supported by chemical analyses of the glands. Even allowing the wide margin of 0.5 to 1.5 only half the total figures fall within these limits. Of considerable interest, however, is the wide divergence from the normal of the group of confusional insanity. This has the low ratio of 0.35 due to the low proportion of phosphatide in the adrenal which, as stated previously, probably results from the toxic condition associated with these cases.

#### SUMMARY.

Analyses of the total alcohol-ether soluble extractives of the adrenals and gonads of 38 patients in mental hospitals have been carried out by special methods.

The percentages of total fatty acid, lecithin, sphingomyelin, free and ester cholesterol and free fatty acid have been determined and the average results for various mental and age groups are given. The percentage amount of fatty extractives from the gonads was found to show much less variation than that from the adrenals. The lowest adrenal content was found in dementia praecox and the highest in senile dementia.

Low values of phosphatide were obtained in the adrenals of the confusional group, and high figures were found in the adrenals of epileptics.

The relative proportions of the total cholesterol to the total phosphatide varied greatly in the cases examined, and the ratio of these proportions in the pairs of glands differed from unity in a marked degree so that the evidence from quantitative chemical analysis of the fatty constituents does not support the view that the lipoids of the gonads are elaborated by and transported directly to these glands from the adrenal cortex.

The above investigations have been carried out by the aid of a scholarship in the Research Laboratories of the Joint Board of Research for Mental Diseases of the University and City of Birmingham.

I wish to express my indebtedness to the Director, Dr F. A. Pickworth, for constant assistance during the planning and conducting of the research.

My thanks are also due to Dr T. C. Graves, the Medical Superintendent of the Birmingham Mental Hospitals, and to the Medical Officers of the Institutions, for the clinical notes on the cases.

#### REFERENCES.

- Bauman and Holly (1923). *J. Biol. Chem.* **55**, 457.  
Biedl (1910). Verhandl. VIII Internat. Zool. Kongress.  
Caminade (1922). *Bull. Soc. Chim. biol.* **4**, 601.  
Chauffard (1918). *Compt. Rend. Soc. Biol.* **91**, 87.  
Chauffard, Laroche and Grigaut (1920). Congress français de méd.  
Elliott and Armour (1911). *J. Path. Bact.* **15**, 481.  
Elliott and Tuckett (1906). *J. Physiol.* **34**, 332.  
Fenger (1916). *J. Biol. Chem.* **27**, 303.  
Levene (1916). *J. Biol. Chem.* **24**, 69.  
Levene and West (1916). *J. Biol. Chem.* **24**, 41.  
Mair (1912). *J. Path. Bact.* **18**, 185.  
Mott (1920). *Proc. Roy. Soc. Med.* **13**, 25.  
Mott and Hutton (1923). *Brit. Med. J.* **ii**, 234.  
Mott and Kojima (1917). *Proc. Roy. Soc. Med.* **10**, 188.  
Mueller (1917). *J. Biol. Chem.* **30**, 39.  
Papea and Eustatziu (1925). *Compt. Rend. Soc. Biol.* **93**, 747.  
Rosenheim and Tebb (1909). *J. Physiol.* **38**, Proc. liv.  
Sorg and Jaffé (1924). *Centr. allgem. Path.* **35**, 353.  
Windaus (1910). *Z. physiol. Chem.* **65**, 110.

# CXXXV. VITAMIN A FORMATION. THE FEEDING OF ETIOLATED WHEAT SHOOTS TO RATS KEPT IN DARKNESS.

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*(Report to the Medical Research Council.)*

*(Received June 30th, 1928.)*

ALTHOUGH an accumulation of evidence [Coward and Drummond, 1921; Coward, 1923, 1, 1925; Harrow and Krasnow, 1922, 1924; Karshan, Harrow and Krasnow, 1927; Dye, Medlock and Crist, 1927; Heller, 1928] has pointed to some relation between photosynthesis and abundance of vitamin A in plant tissues, ample proof has been afforded that the feeding of etiolated wheat shoots grown in the dark may effectively repair a deficiency of the vitamin [Wilson, 1922; Coward, 1927; Moore, 1927]. Such evidence would suggest that the action of light is not an essential for the formation of the vitamin, serving only to accelerate processes which occur spontaneously in the dark, but before this conclusion can be drawn it is necessary to decide whether such short periods of illumination (of the shoots or the experimental animal) as are admitted in the normal technique of rat feeding may have played any part in the synthesis.

It may be allowed at once that should the appearance of chlorophyll be adopted as the sole criterion of photosynthesis further research on the point would be unnecessary, since with a hungry rat the shoots need remain uneaten and exposed for only a short period, but such a criterion cannot well be justified. Experience has shown that a very short exposure to ultra-violet irradiation will suffice for the production of vitamin D from ergosterol, and it is conceivable that equally short exposures to visible light might effect the synthesis of vitamin A in the etiolated plant before there is time for noticeable "greening" to take place. Apart from action on the plant tissues, an additional possibility, as in the case of the production of vitamin D, is the action of light upon the animal *in vivo*, after the absorption of a provitamin derived from the shoots.

In control experiments it has been found that ungerminated wheat seeds, fed to rats in numbers equal to effective doses of etiolated shoots, do not noticeably delay the decline and death of the animals, even when no precautions are taken to shield the seeds and animals from light. During growth in the dark, therefore, at least one step in the synthesis of the vitamin is accomplished. It remains to be decided whether the vitamin is completely

synthesised in this one step or whether a precursor is first formed by non-photosynthetic processes and subsequently activated by very short periods of illumination.

Rosenheim and Drummond [1920] once suggested that vitamin A might be produced by the action of light on a pigment of the carotinoid type, and it has been shown by Coward [1923, 2] that during the growth of the etiolated wheat shoot there is a considerable synthesis of the pigment xanthophyll. Recently [Rosenheim, 1927] the trend of speculation has been centred more on ergosterol than on the carotinoids as a precursor of vitamin A, but the possibility of any mechanism of a similar "two stage" nature emphasises the need of caution before it is finally assumed that light plays no part in the production of the vitamin.

The experiments to be described were planned to test the vitamin A content of etiolated wheat shoots under "dark room" conditions, a minimum intensity of red light being admitted to allow the necessary manipulations of the shoots and animals.

#### EXPERIMENTAL.

The experiments were conducted in a darkened room, warmed by a covered gas fire and ventilated by shafts, which were arranged vertically and blackened internally to prevent reflection of light. Entrance to the room could be effected by means of a double door. For constant illumination a small ruby lantern was provided; the animals and their food could barely be distinguished by its light when the eye had become adjusted to darkness. For the purpose of collecting shoots and preparing diet somewhat brighter illumination was required, and since electric light was not available, light was admitted when necessary through a small ruby glass window protected by a shutter. The shoots were grown, as in the previous experiment [Moore, 1927], upon moist sand for a period of 10 days. To avoid unnecessary exposure to light from the ruby window the trays were covered with a screen of black calico.

For testing the activity of the shoots two groups (A and B) of four young piebald rats each and two groups (C and D) of four young Wistar albino rats each were used. To all four groups a standard basal diet prepared by the British Drug Houses was supplied, supplemented in all cases by an allowance of 0.75 cc. of marmite extract per animal daily. In the case of groups B and C, used to test the main question of the vitamin A content of the shoots, and of group A, the negative control group receiving seeds, the diet was further supplemented by an allowance of one drop of "radiostol" (a preparation of 0.1 % of irradiated ergosterol in arachis oil) per animal daily. Group D was used to test the effect of the omission of vitamin D from the diet when etiolated shoots were supplied from the commencement of the experiment, and the "radiostol" was accordingly omitted.

As shown by the accompanying growth curves all four animals in Group A declined in weight after a period of 7 weeks on the experimental diet, signs

of xerophthalmia being evident. Upon the addition to the diet of 30 wheat seeds per animal daily no response was obtained, and the animals died. In the case of groups B and C, which received respectively 30 and 15 etiolated wheat shoots per animal daily after growth had slackened, a slow resumption

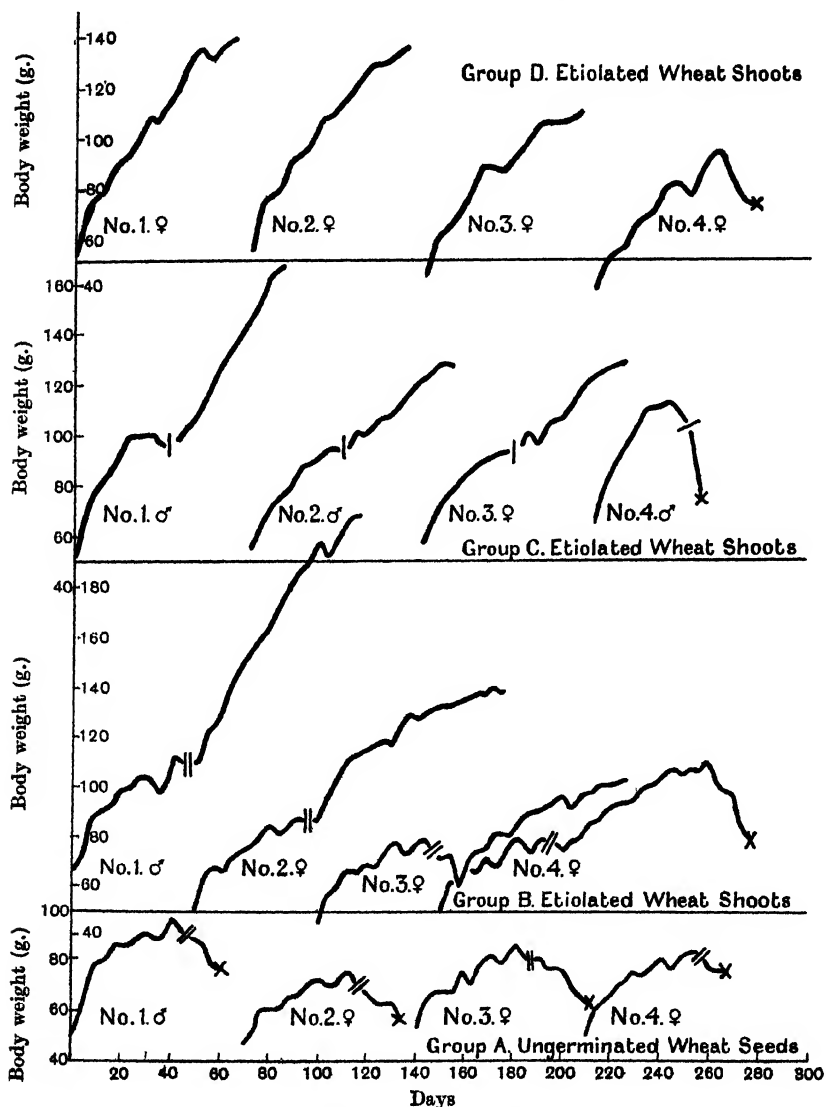


Fig. 1. Growth curves.

in growth was obtained in six animals. One of the remaining two animals (Group B, No. 4) after a preliminary response during several weeks declined slowly and died from intestinal stoppage; the other (Group C, No. 4) had



probably already reached a point of rapid decline in which the feeding of a source of such low activity as the etiolated shoot was ineffective. In the case of Group D, 15 shoots per rat daily were allowed from the commencement of the experiment, with a view to deciding whether vitamin D could be made to become the limiting factor in growth when vitamin A was supplied by etiolated shoots, and whether the "radiostol" supplied in the preceding experiments had been an essential or merely precautionary measure. Only one rat showed an early decline, and death was not prevented by the administration of "radiostol" in large doses. The remaining rats attained weights at which some slackening in growth might well have been anticipated from the behaviour of rats receiving both shoots and "radiostol," and the experiment was discontinued.

#### DISCUSSION.

Since the results obtained under "dark room" conditions differed in no way from those obtained under ordinary conditions of illumination, it is obvious that should light be concerned in the synthesis of the vitamin the exposures necessary are extremely short, and that red light is effective. A strong temptation must exist to assume that the synthesis of the vitamin is independent of light, but this must be qualified by a consideration of the extreme sensitivity of etiolated plant tissues to diffuse light. To illustrate the point it may be mentioned that while the exposures to red light permitted in the experiment did not cause noticeable "greening" of the shoots, inadvertent exposure to this light for some hours of a trial batch of shoots, before the commencement of the actual experiment, resulted in quite definite greening. If the complicated chlorophyll molecule can be synthesised after such short and diffuse exposure it must remain a possibility, though no more than a possibility, that vitamin A may be synthesised during even shorter exposures.

In general the trend of the experiments has been to confirm the view that the etiolated wheat shoot is a definite but unsatisfactory source of vitamin A. While xerophthalmia is cured and a slow resumption in growth is promoted, rats receiving etiolated shoots are often lean and rough coated, and particularly liable to sneezing and lung troubles. This may probably be attributed simply to the low content of vitamin in the shoots, but it must be remarked that even when comparatively large doses of shoots are supplied, growth and condition seem to be no better than with smaller doses. Thus Coward [1927] has found that five etiolated wheat shoots are sufficient to procure slow growth; the effect of feeding 15 or 30 shoots would not appear to produce better results. In our experiments animals receiving 15 shoots daily grew as well as, or better than, rats receiving 30 shoots, and it is possible that the inclusion of large amounts of moist vegetable tissue in the diet of rats is harmful. This possibility was supported by the finding of large amounts of undigested fibres in the gut of the rat (Group B, No. 4) which died from some intestinal disturbance, but no systematic enquiry into the matter has been made.

## SUMMARY.

Etiolated wheat shoots tested upon rats under conditions involving the minimum of red light illumination consistent with the feeding and handling of the animals were found to be effective as a source of vitamin A, supporting the conclusion that light is not essential during any stage of the formation of the vitamin from the seed.

My sincere thanks are due to Dr L. J. Harris for valuable advice and criticism, and to Mr Alfred Ward for technical assistance.

## REFERENCES.

- Coward (1923, 1). *Biochem. J.* 17, 134.  
—— (1923, 2). *Biochem. J.* 17, 145.  
—— (1925). *Biochem. J.* 19, 500.  
—— (1927). *J. Biol. Chem.* 72, 781.  
Coward and Drummond (1921). *Biochem. J.* 15, 530.  
Dye, Medlock and Crist (1927). *J. Biol. Chem.* 74, 95.  
Harrow and Krasnow (1922). *J. Metab. Res.* 2, 401.  
—— (1924). *Proc. Soc. Exp. Biol. Med.* 21, 232.  
Heller (1928). *J. Biol. Chem.* 76, 510.  
Karshan, Harrow and Krasnow (1927). *Proc. Soc. Exp. Biol. Med.* 24, 765.  
Moore (1927). *Biochem. J.* 21, 870.  
Rosenheim (1927). *Biochem. J.* 21, 386.  
Rosenheim and Drummond (1920). *Lancet*, i, 862.  
Wilson (1922). *J. Biol. Chem.* 51, 455.

## CXXXVI. THE EFFECT OF ARSENIC UPON SOME OXIDATION-REDUCTION SYSTEMS.

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*(Received July 2nd, 1928.)*

IN 1887 Jonathan Hutchinson pointed out that persons who took arsenic for long periods were liable to cancer of the skin. At the present time records of about forty cases of this form of cancer of the skin can be collected from the literature [Kennaway, 1925]: in a large proportion of these the arsenic was taken in the form of potassium arsenite (Fowler's solution). Now, cancer can be produced experimentally by the action of another class of chemical compounds, namely, certain constituents, as yet unidentified, of coal-tar [Bayon, 1912], shale oil, and some petroleums [Leitch, 1922, 2, 1924]. The evidence available suggests that the active compounds in these complex mixtures are (1) of high boiling point, (2) neutral, (3) free from nitrogen and sulphur, and it seems probable that they are hydrocarbons of high molecular weight [Bloch, 1922; Kennaway, 1924]. Hence cancer of the skin can be induced by two wholly different chemical agents, namely, by arsenites and by high-boiling hydrocarbons, which seem to have no characters in common. The experiments described below represent an attempt to investigate this anomaly.

In a case in which arsenic cancer develops there is usually a prolonged period of hyperkeratosis of the palms and soles before any malignant change occurs. As an actual instance one may take that recorded by Semon [1922]. The patient took arsenic from 1894 to 1901: in the latter year hyperkeratosis was present and this persisted without further intake of arsenic until the date of observation (1922). In any attempt to investigate the part played by arsenic in such a case one requires to have some idea of the concentration of it in the skin during the precancerous stage. There is in the literature a very large number of data on the quantities of arsenic found (1) in the hair of persons who had taken known amounts of Fowler's solution over short periods, and (2) in the hair of rats and the feathers of fowls to which arsenic had been administered [Report of Royal Commission, 1903]. We have found only one set of analyses on the tissues of a human subject who could be considered to be in any stage of the precancerous condition. This was recorded by Dixon Mann [1914] who found, in a case in which there was pronounced hyperkeratosis, that "the true skin contained an unweighable amount, whereas the horny scales yielded equal to 0.013 %, and the hair equal to 0.008 % of arsenious

oxide. The nails probably contained still more, judging from the deposits yielded by the small available weights of nail-clippings."

As we were unable to find any fuller account of the case of hyperkeratosis, these scanty data had to serve as the basis of the experiments described in the present paper. We tested the effect of the concentrations of arsenic found by Dixon Mann (*i.e.* about 10 mg. arsenic in 100 cc.) upon three oxidation-reduction systems, namely:

- (1) hypoxanthine and the xanthine oxidase of rat and mouse skin;
- (2) acetaldehyde and colloidal platinum;
- (3) acetaldehyde or propaldehyde, glycine and phosphate.

Unfortunately we have so far been unable to detect with any regularity the oxidation-reduction changes which must undoubtedly occur in human skin [Kaye, 1924; Walker, 1925]. A large number of experiments upon extracts and suspensions of this tissue, obtained generally from freshly excised breasts, with various combinations of methylene blue, indigo carmine, hypoxanthine, acetaldehyde and sodium succinate have not given reduction which was sufficiently constant to allow the effect of arsenite upon it to be demonstrated. Decoloration was often absent, or very irregular in a series of tubes, or extremely slow. Various causes may contribute to this; human skin as obtained in the ordinary way consists very largely of the inert connective tissue of the dermis and is excessively tough for thorough mincing; and possibly xanthine oxidase, which has a very capricious distribution in different species and tissues [Morgan, 1926], is absent; or the insolubility of the substance in skin giving the nitroprusside reaction [Walker, 1925] may render the technique used in those experiments unsuitable. Further experiments are in progress by which it may be possible to obtain some indication of the oxidations in human skin and of the effect of arsenic upon these. In the meantime we have used the skin of the rat and mouse.

Spitzer [1898] showed that some tissues convert  $\text{As}_2\text{O}_3$  into  $\text{As}_2\text{O}_5$ ; that is, the  $\text{As}_2\text{O}_3$  in this case acts as an oxygen acceptor. Our experiments were begun with the expectation that arsenic trioxide would act thus as an oxygen acceptor under other conditions also, and might bring about reduction in the same manner as do hypoxanthine or aldehyde when in the presence of xanthine oxidase or colloidal platinum; but the results showed at once that arsenite had an inhibitory instead of an accelerating action. It was found most convenient to make the solutions of arsenite and arsenate of such strength that they contained arsenic in about ten times the concentration found by Dixon Mann: if one then added the solution to the mixture in the Thunberg tube in such amounts as to make up one-tenth of the whole, the percentage of arsenic found in the hyperkeratotic tissues was provided. The arsenite solutions used were derived from three sources.

(1) A preparation of sodium arsenite, obtained from chemical manufacturers, stated to be  $\text{NaAsO}_2$ . A solution of 0.173 % strength contains 0.1 % As.

(2) In order to obtain a very pure material,  $\text{As}_2\text{O}_3$  was sublimed and a weighed amount of the sublimate dissolved with the amount of  $\text{NaOH}$  calculated to give  $\text{NaAsO}_2$  (i.e. 0.2653 g.  $\text{As}_2\text{O}_3$  dissolved with 26.6 cc. 0.1  $N$   $\text{NaOH}$  and made up to 200 cc.; this contains 0.1 %  $\text{As}$ ).

(3) Fowler's solution was used in order to test the action of arsenic in the actual form which has been taken in most cases of arsenic cancer.

Fowler's solution (liquor arsenicalis) consists of

Arsenious oxide	...	...	...	...	10 g.
Potassium carbonate	...	...	...	...	10 g.
Compound tincture of lavender	...	...	...	...	30 cc.
Water to	...	...	...	...	1 l.

It thus contains 1.0 %  $\text{As}_2\text{O}_3$  and 0.75 %  $\text{As}$ . This solution contains excess of alkali, the calculated amount of  $\text{K}_2\text{CO}_3$  to form  $\text{KAsO}_2$  being 7 g.

The arsenate solution used contained 0.25 %  $\text{Na}_2\text{HAsO}_4$ , obtained from chemical manufacturers; this is equivalent to 0.1 %  $\text{As}$ .

#### XANTHINE OXIDASE OF RAT AND MOUSE SKIN.

A large number of preliminary tests were required in order (1) to decide upon a technique for obtaining sterile extracts of skin; filtration through a candle was found to give inactive fluid; (2) to ascertain the optimum proportions of hypoxanthine and methylene blue; too great an excess of the former inhibits the reaction [see Dixon and Thurlow, 1924].

The following method was finally adopted. (The phosphate solution referred to throughout this paper was Sørensen's mixture of  $p_{\text{H}}$  7.5.)

(1) The extracts of skin were prepared by several different methods.

(a) A rat or several mice were killed, the bodies epilated with  $\text{Na}_2\text{S}$  and washed in running water. The skin was removed, minced and placed in phosphate solution (1 cc. to 1 g.) either saturated with toluene, or containing 0.1 % acriflavine. The mixture was shaken for 15 minutes, and after this stage was brought into contact with sterile materials only. It was strained through cotton wool or linen and centrifuged, and the supernatant fluid was kept in the ice-chest overnight in the presence of toluene when flavine was not used.

(b) The removal of hair at the start of course assists greatly in sterilisation, but the smell of sulphide was found to be perceptible in epilated skin even after very prolonged washing, and it was feared that the compound might bring about reduction. Hence in some cases the body was soaked in 5 % lysol, washed in water for an hour and the unepilated skin minced and treated further as stated above.

(2) To avoid sterilisation by heat with possible decomposition, 10 mg. hypoxanthine were dissolved in 0.75 cc. 0.1  $N$   $\text{NaOH}$  and 9.25 cc. boiled phosphate solution by warming in a sterile tube; a few drops of toluene were added, the whole was shaken and allowed to stand for some hours before use. It was found very useful, when all the materials for an experiment were ready, to make a rough preliminary aerobic test with amounts of hypoxanthine in the

ratio 1 : 2 : 3 : 4, in order to ascertain the optimum, which varies with different skins.

(3) The methylene blue (1 in 5000 in phosphate solution) and arsenic solutions were placed first in the Thunberg tubes which were then wool-plugged and autoclaved, the taps being sterilised separately and touched with sterile vaseline before insertion. The skin extract, hypoxanthine, and two drops of toluene were then added and the tubes evacuated with a Fleuss pump. The side tubes were wool-plugged and the taps not turned on until the pressure had been lowered, so that no contamination by entry of air could occur. The tubes were in some cases simply evacuated, in others refilled twice with nitrogen, and were then incubated at 40°.

Details of five experiments with skin are given in Tables I-III. The results show that arsenic in the concentrations found by Dixon Mann (about 10 mg. in 100 cc.) prolong the reduction time five to tenfold, while smaller amounts (2 to 7.5 mg. in 100 cc.) cause slowing to two to five times the control time. Although the delay with the larger amounts of arsenic is obvious, it is difficult to record this in exact figures, because the last stages of the reduction are so drawn out that it is not easy to decide when the last trace of blue disappears, and often the process comes to a standstill when almost complete (see for instance Table III). No doubt it would be better to compare the times of half-reduction.

Arsenate (equivalent to 10 mg. As in 100 cc.) was tested in four experiments only; in three of these the reduction time was the same as in the control (Table II, Extract B); in the fourth there was a slight retardation (from 8 to 11 mins.; Table II, Extract A).

Table I.

Tube	Rat skin extract (cc.)	Methylene blue 1 in 5000 (cc.)	Hypo-xanthine 1 mg. in 1 cc. (cc.)	NaAsO <sub>2</sub> from sublimed As <sub>2</sub> O <sub>3</sub> (cc.)	Phosphate solution (cc.)	Reduction time (mins.)	Approx. conc. of arsenic (mg. in 100 cc.)
1	0.4	0.45	0.1	—	1.0	c. 6	—
2	0.4	0.45	0.1	—	1.0	c. 6	—
3	0.4	0.45	—	—	1.0	? begun 60	—
4	0.4	0.45	0.1	0.2	0.8	c. 42	10
5	0.4	0.45	0.1	0.2	0.8	c. 60	10
6	0.4	0.45	0.1	0.1	0.9	a.c. 19 then very slow	5
7	0.4	0.45	0.1	0.1	0.9	Ditto	5
8	0.4	0.45	0.1	0.07	1.0	c. 14	3.5
9	0.4	0.45	0.1	0.07	1.0	c. 16	3.5
10	0.4	0.45	0.1	0.04	1.0	c. 7	2
11	0.4	0.45	0.1	0.04	1.0	c. 14	2

Cultures in broth from all tubes were sterile.

The ratio of hypoxanthine to methylene blue is 3 mols. : 1 mol.

In this and subsequent tables c. = complete; a.c. = almost complete.

The possibility must be considered that the arsenical solutions act thus in virtue of their alkalinity: but (1) the addition of the minute amount of alkali to the large volume of buffer produces no change detectable by neutral

Table II.

Tube	Rat skin extract (cc.)	Methylene blue 1 in 5000 (cc.)	Hypo- xanthine 1 mg. in 1 cc. (cc.)	NaAsO <sub>2</sub> 0.173 % (cc.)	Na <sub>2</sub> HAsO <sub>4</sub> 0.25 % (cc.)	Phos- phate solution (cc.)	Reduction time (mins.)	Approx. conc. of arsenic (mg. in 100 cc.)
<b>Extract A:</b>								
1, 2	0.5	0.45	0.07	—	—	1.0	c. 8, 8	—
3, 4	0.5	0.45	—	—	—	1.0	Nil 60	—
5, 6	0.5	0.45	0.07	0.2	—	0.8	a.c. 60	10
7, 8	0.5	0.45	0.07	0.4	—	0.6	Advanced 60	20
9, 10	0.5	0.45	0.07	—	0.2	0.8	c. 11, 11	10
11, 12	0.5	0.45	0.07	—	0.4	0.6	c. 11, 11	20
<b>Extract B:</b>								
1, 2	0.5	0.45	0.04	—	—	1.0	a.c. 89	—
3	0.5	0.45	—	—	—	1.0	Nil 89	—
NaAsO <sub>2</sub> from sublimed As <sub>2</sub> O <sub>3</sub>								
4, 5	0.5	0.45	0.04	0.2	—	0.8	Nil 89	10
6, 7	0.5	0.45	0.04	0.1	—	0.9	Nil 89	5
Fowler's solution 1/10								
8, 9	0.5	0.45	0.04	0.2	—	0.8	Nil 89	10
10, 11	0.5	0.45	0.04	—	0.2	0.8	a.c. 89	10

Cultures in broth from all tubes in series A were sterile; tubes in series B were not tested.

The ratio of hypoxanthine to methylene blue is, with Extract A, 2.1 mols. : 1 mol., and with Extract B, 1.2 mol. : 1 mol.

Table III.

Tube	Mouse skin extract (cc.)	Methylene blue 1 in 5000 (cc.)	Hypo- xanthine 1 mg. in 1 cc. (cc.)	Fowler's solution 1 in 10 of phosphate solution (cc.)	Phos- phate solution (cc.)	Reduction time (mins.)	Approx. conc. of arsenic (mg. in 100 cc.)
<b>Extract A (3 skins):</b>							
1	0.5	0.45	0.15	—	1.0	25	—
2	0.5	0.45	0.15	—	1.0	28	—
3	0.5	0.45	0.15	0.2	0.8	70	7.5
4	0.5	0.45	0.15	0.2	0.8	85	7.5
NaAsO <sub>2</sub> 0.173 %							
<b>Extract B (3 skins):</b>							
1	0.4	0.45	0.15	—	1.0	22	—
2	0.4	0.45	0.15	—	1.0	20	—
3	0.4	0.45	—	—	1.0	Nil in 90	—
4	0.4	0.45	0.15	0.1	0.9	a.c. 49	5
5	0.4	0.45	0.15	0.1	0.9	a.c. 90	5
6	0.4	0.45	0.15	0.1	0.9	77	5
7	0.4	0.45	0.15	0.07	1.0	43-49	3.5
8	0.4	0.45	0.15	0.07	1.0	43	3.5
9	0.4	0.45	0.15	0.07	1.0	49	3.5
10	0.4	0.45	0.15	0.04	1.0	43-49	2
11	0.4	0.45	0.15	0.04	1.0	a.c. 90	2
12	0.4	0.45	0.15	0.04	1.0	43	2

The ratio of hypoxanthine to methylene blue is 4.5 mols. : 1 mol.

Last stages of reduction in B tubes 4-12 were very slow and irregular, hence exact times of reduction were difficult to fix. Contents of all B tubes showed same tint on addition of neutral red.

red (Table III); (2) in earlier experiments in which the arsenical solutions were neutralised first of all the same changes were observed; (3) xanthine oxidase is not sensitive to changes of  $p_H$  from 5.8 to 9.0 [Dixon and Thurlow, 1924]; (4) with colloidal platinum, retardations of the same type are produced by neutralised arsenical solutions in the presence of 0.01 *N* NaOH.

#### COLLOIDAL PLATINUM.

Bredig and Sommer [1910] showed that platinum and other metals in the colloidal state could bring about the reduction of methylene blue by aldehydes in an alkaline medium. Their colloidal solutions were made by electrical dispersion. In the experiments recorded here, preparations<sup>1</sup> made by reduction were used. Acetaldehyde was taken throughout as oxygen acceptor; hypoxanthine does not act in conjunction with platinum. The alkaline reaction required was produced by adding to the mixture in the Thunberg tube one-tenth of its volume of 0.1 *N* NaOH. The ratio of acetaldehyde (0.33 or 1.0 cc. of 2 vols. % solution) to methylene blue (4 cc. of 1 in 5000) was from 56 to 169 times the theoretical equimolecular proportion; in the experiments of Bredig and Sommer this ratio is greater still, being 1000 : 1 or more.

Table IV.

Tube	Colloidal Pt 0.1 % (cc.)	Neutral CH <sub>3</sub> CHO 2 vols. % (cc.)	Methylene blue 1 in 5000 (cc.)	NaOH 0.1 <i>N</i> (cc.)	H <sub>2</sub> O (cc.)	Neutral NaAsO <sub>2</sub> 0.173 % As (cc.)	Neutral Na <sub>2</sub> HAsO <sub>4</sub> 0.25 % As (cc.)	Re- duction time (mins.)
1	1.0	1.0	4.0	0.7	0.7	—	—	6
2	1.0	1.0	4.0	0.7	0.7	—	—	5
3	1.0	1.0	4.0	0.7	0.7	—	—	6
4	1.0	1.0	4.0	0.7	—	0.7	—	267
5	1.0	1.0	4.0	0.7	—	0.7	—	a.c. 267
6	1.0	1.0	4.0	0.7	—	0.7	—	a.c. 267
7	1.0	1.0	4.0	0.7	—	—	0.7	7
8	1.0	1.0	4.0	0.7	—	—	0.7	8
9	1.0	1.0	4.0	0.7	—	—	0.7	7

Tubes evacuated, filled with nitrogen, and incubated at 40°.  
For concentrations of arsenic see Table V.

In all, 18 experiments were carried out with colloidal platinum. Of 15 experiments with arsenite, 13 gave retardation of the type summarised in Table V; of the remaining two, in which the ratio of methylene blue to platinum was high, one gave very slow and irregular results, and in the other, the arsenic in the lower concentrations (1.0 and 0.1 mg. per 100 cc.) caused acceleration, for which no explanation could be found. A detailed record of an experiment is given in Table IV, and a summary of a number of results in Table V.

The results given in Table V show that arsenic, as arsenite, when in concentrations of the order observed by Dixon Mann (*e.g.* 9.5 mg. in 100 cc.) retards the reduction of methylene blue by platinum and aldehyde by as much as twenty- or forty-fold; but when the amount of arsenic is reduced to

<sup>1</sup> Obtained from the Crookes Laboratories, London.



one-tenth of this (*i.e.* to 0.95 mg. in 100 cc.) the reduction time is prolonged not more than  $2\frac{1}{2}$  times; and at still lower concentration (0.095 mg. in 100 cc.) the retardation is slight or absent, and in one case there was even an acceleration (to 0.85 of the control time). Under the conditions adopted, the platinum-aldehyde system is more affected by a given concentration of arsenite than is the xanthine oxidase of rat and mouse skin. If the reaction be slowed by increasing the ratio of methylene blue solution to those of platinum and aldehyde from 4 : 1 to 12 : 1 the general character of the results is not altered.

Table V.

Colloidal platinum and arsenite.			
CH <sub>3</sub> CHO Pt	Ratio Methylene blue	Arsenic in 100 cc. in whole mixture (mg.)	Mean reduction time in controls (mins.)
	1 : 4	4.3	5.5
	1 : 4	9.5	5.6
	1 : 12	9.5	Less than 1350
	1 : 4	9.5	5.0
		0.95	
	1 : 12	0.095	82.0
		0.95	
	1 : 4	0.95	5.0
		0.095	
		9.5	
	1 : 12	0.95	67.5
		0.095	
		9.5	
	1 : 4	0.95	6.0
		0.095	
		0.98	
	1 : 8	0.098	71.0
CH <sub>3</sub> CHO : Pt : MB		4.3	23.5
1 : 4 : 16			

Mean reduction  
time in presence  
of arsenic if control  
reduction time = 1  
(Partial in 1350)  
More than 60

The figures in the first column indicate the relative volumes of the following solutions:

CH<sub>3</sub>CHO = 2 vols. % CH<sub>3</sub>CHO in water neutralised with NaOH.

Pt = 0.1 % colloidal platinum.

MB = 1 in 5000 methylene blue in water.

In all the experiments recorded except the last the volumes of aldehyde and platinum solutions were equal.

Arsenite solution used was neutralised 0.173 % NaAsO<sub>2</sub> (= 0.1 % As).

Tubes were evacuated, filled with nitrogen and incubated at 40°.

Thus the platinum-aldehyde system seems to be especially sensitive to concentrations of arsenic of an order not less than those observed in hyperkeratotic skin. The lower percentages tested (0.95 to 0.095 mg. per 100 cc.) are similar to those found in the hair of persons who have been taking medicinal doses of Fowler's solution for 1 or 2 months (2.0 to 0.16 mg. per cent.) [Report of Royal Commission, 1903].

Arsenate (equivalent to 10 mg. As in 100 cc.) was tested in five experiments of which one gave very discordant results in duplicate tubes, and was rejected. The remaining four experiments gave mean reduction times of 12.2' in the controls and 16.3' in the presence of arsenate, *i.e.* a retardation of 25 % only.

## THE GLYCINE-ALDEHYDE-PHOSPHATE SYSTEM.

A series of experiments was made also with the glycine-phosphate-aldehyde system of Haehn and Pülz [1924]. The action of this is illustrated by the experiments recorded in Table VI which shows (Exp. 1) that the omission of any one of the three constituents renders the course of the reaction extremely slow, or stops it altogether. Exp. 11 shows further that increase of phosphate does not compensate in any way for absence of the amino-acid. The results obtained with the Haehn system have been irregular: the identity of the reduction times in triplicate tubes seen in Exp. 11 (Table VI) is exceptional. More often there is a fairly wide range of variation, and we adopted the method of putting up 3, 4 or 5 identical tubes of each mixture in order to eliminate

Table VI.

Tubes <i>Exp. 1</i>	H <sub>2</sub> O (cc.)	Glycine 0.125 <i>M</i> in H <sub>2</sub> O (cc.)	Phosphate (cc.)	CH <sub>3</sub> CHO 2 vols. % in H <sub>2</sub> O (cc.)	Methylene blue, 1 in 5000 in H <sub>2</sub> O (cc.)	Reduction time
1	—	2.5	2.5	0.1	0.25	c. 30'
2	—	—	5.0	0.1	0.25	a.c. next day
3	2.5	2.5	—	0.1	0.25	Partial next day
4	—	2.5	2.5	—	—	Partial next day
<i>Exp. 11</i>		Glycine 0.125 <i>M</i> in phosphate		CH <sub>3</sub> CHO 0.2 vols. % in phosphate	Methylene blue, 1 in 5000 in phosphate	NaAsO <sub>2</sub> neutral 0.173 %
1, 2, 3	0.5	2.5	—	1.0	0.25	—
4, 5, 6	—	2.5	—	1.0	0.25	—
7, 8, 9	0.5	—	2.5	1.0	0.25	—
10, 11, 12	—	—	2.5	1.0	0.25	—
						c. 20, 20, 20' c. 25, 25, 25' Nil in 24 hours Nil in 24 hours

Phosphate = Sorensen's solution of  $p_H$  7.5. Tubes evacuated, filled with N<sub>2</sub>, and incubated at 40°. Tubes 4-6 and 10-12 in Exp. 11 contain 0.012 % As.

Table VII.

	H <sub>2</sub> O (cc.)	Glycine 0.125 <i>M</i> (cc.)	CH <sub>3</sub> CHO 0.2 vols. % (cc.)	Methylene blue, 1 in 5000 (cc.)	NaAsO <sub>2</sub> neutralised 0.173 % (cc.)	Reduction time (mins.)
<i>Exp. 6</i>	0.5	2.5	2.0	0.25	—	45, 45, 48, 45, 45 mean 45.6
6	—	2.5	2.0	0.25	0.5	57, 53, 57, 59, 61 mean 57.4
<i>Exp. 7</i>	0.5	2.5	1.0	0.5	—	37, 37, 40, 40, 40 mean 38.8
7	—	2.5	1.0	0.5	0.5	43, 40, 43, 43, 46 mean 43.0

Glycine, CH<sub>3</sub>CHO and methylene blue were dissolved in phosphate of  $p_H$  7.5. Tubes evacuated, filled with nitrogen and incubated at 40°.

these irregularities as far as possible by averaging. Two typical examples of such results are given in Table VII. Using multiple tubes, 13 experiments in all were made: of these, three were rejected as the results in identical tubes were too divergent to be averaged, and in one of these three the arsenic seemed to accelerate reduction considerably. The remaining ten experiments are summarised in Table VIII. These show that arsenite in

amounts corresponding to the concentrations of arsenic observed by Dixon Mann, or in two or three times that amount, prolongs the reduction time by 18 % on the average. In Exp. 2 (see Table VIII) a retardation of no less than seven-fold was obtained in ten tubes giving concordant readings, but we were unable to repeat this remarkable result which is therefore excluded from the average. The system is evidently very sensitive to small differences in conditions, and this perhaps makes its biological interest all the greater.

Table VIII.

	Mean reduction times (mins.)				Arsenic (mg. in 100 cc.)	
	Control	Sodium arsenite	Control	Sodium arsenate		
Exp. 2	24.0	165.0	—	—	7.4	Propaldehyde
3	28.4	36.4	—	—	7.4	"
4	—	—	54.4	50.6	7.4	"
6	45.6	57.4	—	—	9.5	"
7	38.8	43.0	—	—	11.0	Acetaldehyde
8	67.6	81.0	—	—	20.0	"
9	36.0	40.0	—	—	12.0	"
11	20.0	25.0	—	—	12.0	"
12	23.6	25.6	—	23.0	12.0	"
13	28.6	31.6	—	28.0	11.0	"
Mean omitting Exp. 2	36.1	42.5	35.5	33.8		
Ratio	100	118	100	95		

In all the later experiments the tubes were placed in a dark room during incubation, on account of the possibility that unequal illumination caused irregularities [cf. xanthine oxidase, Bernheim and Dixon, 1928]; but neither this change, nor the substitution of acetaldehyde for propaldehyde, made the results distinctly more constant.

With arsenate three experiments only were carried out; these showed a slight acceleration, amounting to 5 % of the control time, and the system therefore differs in this respect from those containing xanthine oxidase or platinum. But in this system arsenate in sufficient concentration (about 0.05 *M*) can take the place of phosphate [Haehn and Pülz, 1924, p. 90].

#### DISCUSSION.

It is of course obvious that one cannot with any certainty compare reactions which take place in a test-tube in an hour or two with changes which in the human skin require many months or years. But the retarding action of arsenite upon some oxidation-reduction systems *in vitro* perhaps justifies the suggestion that arsenic may induce cancer, not by any direct action, but by causing the accumulation in the tissues of some organic compounds which would otherwise be oxidised or reduced to other forms. This supposition would require that the arsenic in the skin should be in the active form of arsenite, whereas we do not actually know whether it is in this form or in that of the inactive arsenate.

In a case of arsenic cancer such as that described above, in which the hyperkeratotic precancerous stage lasted 13 years or more, it is probable that

all the arsenic has disappeared from the tissues long before malignancy develops. There is of course nothing in these experiments, nor so far as we know in any others, to show what is happening in the tissues during this interval. In the case of tar cancer, the period that can elapse between the cessation of action of the first carcinogenic agent and the development of a tumour has been demonstrated experimentally [Leitch, 1922, 1]. The same process is seen when a cancer of the skin in a mule-spinner, or of the bladder in a dye-worker, appears long after exposure to the harmful agent has ceased.

These experiments provide another illustration of the difference in biological action between arsenite and arsenate. In this laboratory a cancer of the skin was produced in four months in one mouse out of a series of one hundred by painting with a solution of potassium arsenite containing 0.09 % As [Leitch and Kennaway, 1922]. A comparable experiment with an arsenate solution has lasted at the present time for 14 months without the appearance of any tumours; but of course many more positive results would be required to enable one to draw any conclusion from such parallel experiments. It is uncertain whether the skin of the rat, which furnished many of the solutions of xanthine oxidase used, is susceptible to arsenic cancer. In the only case in which tar cancer has been induced in a rat [Herly, 1926], 1 % of  $\text{As}_2\text{O}_3$  was added to the mixture, but controls with the arsenical solution alone were negative.

#### SUMMARY.

Arsenic in the form of arsenite, when in concentrations similar to those found in the skin in arsenical hyperkeratosis, has a retarding action upon three oxidation-reduction systems, namely (1) hypoxanthine and the xanthine oxidase of rat or mouse skin; (2) acetaldehyde and colloidal platinum; and (3) propaldehyde or acetaldehyde, glycine and phosphate. Arsenates are comparatively inactive. The possible significance of these findings in connection with the nature of arsenic cancer is discussed.

#### REFERENCES.

- Bayon (1912). *Lancet*, ii, 1579.  
 Bernheim and Dixon (1928). *Biochem. J.* **22**, 113.  
 Bloch (1922). *Leeuwenhoek-Vereeniging*, i, 46.  
 Bredig and Sommer (1910). *Z. physikal. Chem.* **70**, 34.  
 Dixon and Thurlow (1924). *Biochem. J.* **18**, 976.  
 Dixon Mann (1914). Forensic medicine and toxicology. 5th ed. (London), p. 491.  
 Haehn and Pütz (1924). *Chem. Zell. Geweb.* **12**, 65.  
 Herly (1926). *J. Cancer Res.* **10**, 102.  
 Kaye (1924). *Biochem. J.* **18**, 1289.  
 Kennaway (1924). *Brit. Med. J.* i, 564.  
 — (1925). *J. Industr. Hyg.* **7**, 69.  
 Leitch (1922, 1). *Brit. Med. J.* ii, 1101.  
 — (1922, 2). *Brit. Med. J.* ii, 1104.  
 — (1924). *Brit. Med. J.* ii, 943.  
 Leitch and Kennaway (1922). *Brit. Med. J.* ii, 1107.  
 Morgan (1926). *Biochem. J.* **20**, 1282.  
 Royal Commission on Arsenical Poisoning (1903).  
 Semon (1922). *Brit. Med. J.* ii, 975.  
 Spitzer (1898). *Pflüger's Arch.* **71**, 596.  
 Walker (1925). *Biochem. J.* **19**, 1085.

## CXXXVII. THE LIBERATION FROM YEAST OF SUBSTANCES GIVING THE NITROPRUSSIDE REACTION.

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THE observations recorded here were made in the course of a study, of which one part is described elsewhere [Barry *et al.* 1928] of the action of arsenic upon oxidation-reduction systems. We endeavoured at first to examine the fixation of arsenic by glutathione which has been described in several papers by Voegtlin and his fellow-workers [1923, 1925]. To this reaction they have assigned great importance in the detoxication of arsenical compounds, and the process should be of considerable interest in connection with arsenic cancer. Our experiments were made with the skin and muscle of the rat, the Jensen sarcoma, the liver of the calf and fowl, and yeast. We could not find the expected constant difference in the strength of the nitroprusside reaction between extracts of these materials which had, or had not, been treated with solutions of arsenites. Evidently the effect upon the nitroprusside reaction of the combination of arsenic with glutathione, which Voegtlin seems to regard as beyond doubt<sup>2</sup>, requires investigation. However, in the course of these experiments we were led to note the liberation by a number of physical and chemical agents of substances giving the nitroprusside reaction, and these observations are recorded here.

### TECHNIQUE.

The experiments, begun with mammalian cells, have been carried on up till now with yeast, a very convenient material. 2 g. compressed yeast were suspended in 20 cc. of fluid, which was either Pasteur's solution, 10 % sucrose or dextrose in water, or distilled water alone. We have not checked every result given below with yeast suspended in each one of these four media, but in general the effects appear to be of the same relative order whichever medium is used. As a rule the order of increasing strength of nitroprusside

<sup>1</sup> Working under a grant from the Fondation Universitaire de Belgique.

<sup>2</sup> Apparently the only direct evidence which Voegtlin and his co-workers [1923] give for the suggestion that "the —SH group of glutathione may be regarded as the so-called arsenic receptor of mammalian protoplasm" consists in the preparation *in vitro* of arsenic compounds of thioglycollic acid. The numerous results showing the detoxication of arsenic *in vivo* by glutathione do not prove the formation in the body of similar compounds.

reaction given by yeast in these various media is as follows: (1) Pasteur's solution, (2) sugar solution, (3) water (*e.g.* Tables I and X). Thus weakly acting reagents, such as 0.05 *N* organic acids, may give at first a negative reaction in the presence of sugar and a positive reaction in its absence; but after some hours' incubation the two suspensions tend to give the same intensity of reaction, no doubt because most or all of the sugar has been removed by fermentation.

In a series of test-tubes were placed 1 or 2 cc. of the suspension and generally an equal volume of the chemical reagent to be tested; or a suspension of yeast was made up in bulk in the solution under test (*e.g.* a saturated salt solution), either with or without the addition of sugar, and from this samples were withdrawn at intervals. The mixtures were incubated at 37° for varying times<sup>1</sup>. For the colour test, 1 cc. of suspension was mixed with ammonium sulphate (crystals, or 0.5 cc. of saturated solution), and four drops of strong ammonia (0.880) and of fresh 1 % sodium nitroprusside solution were added. In every case control tubes were set up and incubated, containing (1) yeast suspension alone, the amount of colour given by this varies considerably with different samples (see below, "Autolysis"); and (2) each reagent, with Pasteur's or sugar solution, but without yeast; for the possible presence in the various reagents, or production from them during incubation, of acetone, must always be considered. In most cases, a sample of the suspension after exposure to a physical or chemical agent was examined with a 2 mm. objective for evidence of cytolysis. The comparative strength of the colours produced by nitroprusside is shown roughly below by + signs; it was found most convenient to describe a certain colour, similar in depth to 0.0005 *N*  $\text{KMnO}_4$ , as " +," and others as fractions or multiples of this (faint +,  $\frac{1}{4}$  +,  $\frac{1}{2}$  +, ++, 4 +, etc.). A standard colour-scale could be used, but this seemed unnecessary for the comparative results recorded here; the colours in many cases fade so quickly that there is no time for any elaborate method.

Different samples of yeast under the action of the same reagents give nitroprusside reactions of different degrees of intensity; thus with a yeast which gives weak reactions the less strongly acting salts, such as  $\text{Na}_2\text{SO}_4$  or  $\text{KNO}_3$  (Table II), may not cause the production of any colour at all with nitroprusside. Similarly a sample of compressed yeast which is obtained and tested in the morning will be found to give weaker results if it be kept at room temperature till the afternoon and then used again. But the different reagents maintain on the whole their relative positions in the scale of potency; with different yeasts the results shift to and fro over a certain range of intensity.

<sup>1</sup> This temperature is about 10° above the optimum for the growth of yeast, but this does not affect the comparative value of the results, and the investigation brought forward here was concerned with a search for cytolytic agents rather than with the growth of yeast.

## AUTOLYSIS.

The nitroprusside reaction given by many samples of yeast, and by all after sufficient incubation (Table I), may no doubt be attributed to autolysis. The strongest reaction observed in this series is that recorded in Table X. If yeast be autolysed in the ordinary way, by placing a jar filled with it in a warm place for a week or two, the liquefied material will be found to give a very strong colour. Many, if not all, of the positive reactions described below may be due to an acceleration of autolytic processes.

Table I.

Time	In H <sub>2</sub> O	In H <sub>2</sub> O + 10 % sucrose
0 min.	—	—
30 mins.	? ft +	—
60 "	ft +	—
180 "	+ +	+
240 "	+ +	+ +
24 hrs.	+ + +	+ + +

1 g. yeast in 10 cc.

ft + = faint + in this and subsequent tables.

## SOURCES OF ERROR.

1. *False positives.* These would be detected by the control tubes containing no yeast. Thus commercial butyl alcohol cannot be used on account of its acetone content, and sulphites themselves give the reaction. Solutions of caprylic alcohol and of sodium oleate give faint pink or orange colours with nitroprusside, but these are by no means sufficient to account for the reactions observed in the presence of yeast.

2. *False negatives* are more difficult to detect, and may arise from more than one cause. (1) With many reagents the positive reaction does not appear until after a period of incubation of an hour or more (see, for instance, Tables I, V, VI, VII, IX, X, XII) and disappears again later (Tables III, VI, IX, XII). Hence if no test be made during a certain interval of time the reagent may be set down wrongly as negative. After 24 hours' incubation many positive reactions become fainter, or may cease altogether, this being due perhaps to oxidation of glutathione [cf. Harris, 1923]. This source of false negatives can be avoided by testing a sufficient number of times. (2) The reagent itself may interfere with the nitroprusside reaction, and this is more troublesome to detect. If suspensions of yeast be made in saturated solutions of potassium nitrite, and of sodium nitrite, a striking difference will be observed; the former will give a very strong nitroprusside reaction (Table III), the latter none at all however long the incubation be continued. Further tests show, however, that one cannot thereupon reckon sodium nitrite as a negative reagent; one cannot tell whether it does, or does not, liberate reducing substances from yeast because the colour test by which these would be detected is inhibited. It is very difficult, and perhaps not worth while, to devise a perfectly satisfactory means of detecting these false negatives. Two methods

have been used. (a) A suspension of yeast with the reagents in question is heated to  $70^{\circ}$  (see under "Heat" below); if after cooling it then gives as strong a colour as a control, one may suppose there is no interference. Thus suspensions in water, and in saturated  $\text{KNO}_2$  and  $\text{NaNO}_2$ , when heated in this way give a strong colour in the first named, a stronger colour in the second, and no colour at all in the third. This method is not fitted for very volatile substances (acetaldehyde, methyl alcohol) and heating in strong salt solutions is not desirable. (b) A suspension of yeast giving a strong nitroprusside reaction is prepared by the action of phenol, salicylic acid, or thymol (see under "Organic compounds" below); samples are mixed with the reagents under examination, and the colour tests compared with those given by suitable controls. This serves to show the various degrees of inhibitory action exerted by methyl and ethyl alcohols and acetaldehyde and formaldehyde. All the substances recorded as negative below have been examined by one or both of these methods.

Evidently the nitroprusside test is sensitive to the interfering action of many substances, and this should perhaps make one cautious in concluding from a negative test that a tissue extract contains no glutathione. Hurtley [1913], when working with ethyl acetoacetate and acetoacetic acid, found that "quite small quantities of the ester completely inhibited the test when applied to the acid." Probably there are all grades of such interference, ranging from complete suppression, as with saturated  $\text{NaNO}_2$ , to simple decreased sensitivity.

While some substances can thus prevent the production of the colour, others can remove it after its development. If to a few cc. of a suspension in which a strong nitroprusside colour has been produced one adds 1 cc. of toluene, or methyl alcohol, the colour disappears instantly on shaking. One might expect that the precipitates formed when some of the reagents (*e.g.*  $\text{MgCl}_2$ ) mix with those  $((\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_3$ ) required for the test might interfere with the colour by adsorption, but we have not been able to detect this effect except perhaps in some instances ( $\text{HgCl}_2$ ) which have been excluded below.

#### PHYSICAL AGENTS.

*Grinding.* If a 10 % suspension of yeast be ground in a mortar with powdered silica, which is a much more effective abrasive than sand, there is no liberation of substances giving the nitroprusside reaction as long as the mixture is of semi-fluid or creamy consistence, even though the grinding be carried out very vigorously; but when enough silica has been added to make the material semi-solid, a very intense colour is obtained, both in the solid mass and in the supernatant fluid of a portion suspended in water and centrifuged. Microscopical examination of samples taken at intervals during the grinding shows that the disruption of the yeast cell is far more difficult to bring about than one would expect; after prolonged grinding, when the nitro-



prusside reaction is very strong, one still finds large numbers of cells showing no visible sign of injury. However, an interesting difference is shown by staining with watery methylene blue; in the control suspension only a very small proportion of the cells take up the dye, whereas in the ground material in which reducing substances have been liberated a large number of the apparently uninjured cells stain deeply. In view of the very interesting observation of Harris [1923] that frothing in an albumin solution produces a strong nitroprusside reaction, it would perhaps be worth while to try whether the reaction could be obtained from albumin by any process of grinding.

*Heat.* If a 10 % suspension of yeast giving no, or a very faint, nitroprusside reaction be boiled and cooled it will then be found to give a very strong reaction. This change takes place sharply at about 61°; as a rule yeast heated to 59°-60° gives no, or a very faint, colour, but after exposure to 62°-63° the reaction is quite distinct. The change reaches a maximum at 65°-66.5°; yeast exposed to this temperature gives as much colour as does a boiled sample. In all these experiments the time of exposure to any given temperature was 2 minutes. Euler and Lindner [1915] give the following data for the lethal temperature for four different yeasts: (1) 68°, (2) 64°, (3) 62°-64°, (4) 58°-60°. The temperature at which the nitroprusside reaction appeared in our experiments is thus within the range covered by these figures.

*Freezing and thawing.* If a suspension of yeast in test-tubes be frozen solid by immersion of the tube in ice and salt, a strong nitroprusside reaction (3 +) is obtained on thawing both in the whole mixture and in the clear centrifuged fluid; cooling which does not freeze the suspension gives negative results.

*Ultra-violet light.* 5 cc. of a yeast suspension (2 g. in 20 cc. 10 % sucrose) were poured into each of two Petri dishes, which were placed side by side 35 cm. from a mercury vapour lamp; the glass lid was replaced on one of the two dishes, which then served as a control on the warming effect of the lamp. After 50 minutes' exposure, the yeast in the uncovered dish showed a very distinct reaction (1½ + in the whole emulsion and ½ + in the centrifuged fluid), while that from the covered dish gave no reaction at all.

*X-rays.* The effects of both (1) hard and (2) soft rays were tested; neither produced any change detectable by the nitroprusside test. (1) 10 cc. 10 % yeast suspension in a Petri dish were placed for half an hour immediately beneath a large Coolidge tube, with no filter but with a paraffin wax plate 4 cm. thick interposed to prevent heating. The current was 4 ma. at 160 kv. and the distance from the anticathode to the suspension 15.5 cm. (2) A similar suspension was exposed for 19 or 38 minutes immediately below a universal Coolidge tube working at 130 kv. with current of 4 ma., no filter being used.

#### SALTS.

If to a series of test-tubes of yeast suspension one adds more than enough of various salts to produce saturation, or if one suspends yeast in various salt

solutions already saturated, some curious differences in the nitroprusside reactions of the different mixtures can be observed; with some salts a very strong reaction develops, with others none at all, or no more than is given by a control suspension of yeast in water. The table below shows roughly the comparative activity of a number of salts in this respect. The test was made usually after from 1 to 3 hours' incubation.

Table II. 1 g. yeast in 10 cc. of 10 % dextrose or sucrose with salt added to saturation. Incubation at 37° for 1-3 hours.

Salt		Nitroprusside reaction
CaCl <sub>2</sub>	Na thiosulphate	++ to 8+
MgCl <sub>2</sub>	Na salicylate	
Na and K arsenite	Na benzoate	
NaNO <sub>2</sub>	(NH <sub>4</sub> ) <sub>2</sub> NO <sub>2</sub>	+ to 4+
NaCl	NH <sub>4</sub> Cl	
KNO <sub>3</sub>	Y(NO <sub>3</sub> ) <sub>3</sub>	
KH phthalate		
Na <sub>2</sub> SO <sub>4</sub>	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	+ to -
NaH <sub>2</sub> PO <sub>4</sub>	KCl	
NaF	KNO <sub>3</sub>	
Na acetate	KH <sub>2</sub> PO <sub>4</sub>	
Na citrate	BaCl <sub>2</sub>	
(Na and K arsenate)	Ba acetate	
Na <sub>2</sub> HPO <sub>4</sub>	Na oxalate	—
Na tartrate	KClO <sub>3</sub>	

Table III. 1 g. yeast in 10 cc. salt solution without sugar.

		KNO <sub>3</sub>			
		9.5 M	5M		
Time	(mins.)				
0		+++	ft+		
15		+++	ft+		
30		++	ft+		
45		+++	ft+		
60		++	ft+		
90		+	+		
120		++	+		
Solutions saturated at 37°					
g. in 100 cc.*	...	KNO <sub>3</sub> 126	KNO <sub>3</sub> 45.9	NaNO <sub>2</sub> 71.2	Control in water
Molar conc.	...	14.8	4.55	8.4	
Time					
0 min.		+++	-	ft+	-
30 mins.		6+	-	++	ft+
60 "		6+	ft+	+++	ft+
120 "		6+	-	+++	ft+
21 hrs.		-	+	6+	+

\* These data were obtained by weighing 10 cc. of the solution saturated at 37°, and then interpolating for this temperature from the tables in the "Chemiker-Kalender" giving g. salt in 100 g. solution saturated at various temperatures.

It does not seem possible to offer any simple explanation of these results. Possibly all arsenites and salicylates would give similar strong positive results but not many of these salts have been available. The contrast between arsenites and arsenates is very striking in this as in many other biological

reactions; the arsenates, however, have to a certain extent the character of false negatives and have hence been marked with a parenthesis in Table II. The inhibitory action of potassium arsenate is most distinct in the heating test, and that of the sodium salt in the salicylic acid test (see under "Sources of error" above).

One factor which one would expect to influence the results is the molar concentration of the saturated solution but the results show that this is not so.

If one takes any salts, such as  $\text{KNO}_3$ ,  $\text{NaCl}$ , or  $\text{CaCl}_2$ , which when saturated give a strong positive reaction, and prepares a series of dilutions of the saturated solution, one finds that the activity diminishes rapidly as the concentration of the salt is lessened. Thus there is a large difference between 9.5  $M$  and 5  $M$  solutions of  $\text{KNO}_3$  (Table III). A fairly uniform gradation is shown by solutions of  $\text{NaCl}$  (see Table IV) and of  $\text{CaCl}_2$  (see Table V).

The presence of solid salt in a saturated solution seems to increase the effect, but this was tested in one experiment only. In this, 2 g. yeast suspended in 20 cc. 6  $M$   $\text{NaCl}$  (solution A), which is a nearly saturated solution, gave distinctly less colour than did the same quantity of yeast in 20 cc. of water containing an excess (16 g.) of the salt (solution B, see Table IV).

Table IV. 1 g. yeast in 10 cc. No sugar.

Time	$\text{NaCl}$				Solution B saturated + excess salt
	6 $M$	5 $M$	4 $M$	Solution A 6 $M$	
0 min.	ft +	—	—	$\frac{1}{2}$ +	+
90 mins.	ft +	—	—	ft +	$\frac{1}{2}$ +
120 "	ft +	? ft +	—	$\frac{1}{2}$ +	+
135 "	$\frac{1}{2}$ +	ft +	v. ft +		
150 "	$\frac{1}{2}$ +	$\frac{1}{2}$ +	—		
180 "	$\frac{1}{4}$ +	ft +	—		
270 "	$\frac{1}{2}$ +	ft +	—	$\frac{1}{2}$ +	+
22 hrs.	++	+	$\frac{3}{4}$ +	+	+

Table V. 1 g. yeast in 10 cc. No sugar.

Time	$\text{CaCl}_2$			Control in $\text{H}_2\text{O}$
	1 $M$	0.5 $M$	0.25 $M$	
0 min.	? ft +	? ft +	—	
90 mins.	+++	ft +	—	ft +
120 "	+++	ft +	—	—
270 "	++	ft +	—	—
330 "	++	v. ft +	—	—
23 hrs.	+++	+	ft +	—
Time	1 $M$	0.75 $M$	0.5 $M$	
0 min.	$\frac{3}{4}$ +	$\frac{1}{2}$ +	ft +	
60 mins.	++	+	$\frac{1}{2}$ +	
120 "	+++	++	+	
180 "	++	+	$\frac{1}{2}$ +	
20 hrs.	+++	++	+	

Of the inorganic salts tested,  $\text{CaCl}_2$  seems to be the most potent. With the samples of yeast used, a strong reaction was given by a molar solution (about 11 %  $\text{CaCl}_2$ ), and the strength of the salt had to be reduced to 0.25  $M$  before

the treated and control samples ceased to show any difference. With  $\text{KNO}_2$  or  $\text{NaCl}$ , in contrast, the action usually becomes very slight at strengths of 5 or 4  $M$ . One may have here another instance of the great physiological activity of calcium.

#### ACIDS AND BASES.

These have given far more variable results than have any other agents, physical or chemical. With 0.05  $N$  solutions containing 10 % of sugar, one finds as a rule that  $\text{HCl}$  and  $\text{HNO}_3$  give stronger reactions than  $\text{H}_2\text{SO}_4$ , while the organic acids (acetic, tartaric, lactic, oxalic) are less active than the inorganic, and give with many samples of yeast negative results. An example of the type of change observed is shown in Table VI. With 0.1  $N$  solutions in the absence of sugar much stronger reactions are obtained from both inorganic and organic acids. There is an interesting difference between acetic and trichloroacetic acids, the latter being much more powerful than the former.

Table VI.

Time (mins.)	HCl	$\text{H}_2\text{SO}_4$	Acetic acid	Tartaric acid	Oxalic acid	Lactic acid	Control
30	++	++	-	-	+	-	-
60	++	++	ft +	-	+	-	-
120	+++	+++	+	ft +	++	ft +	-
180	+++	+	ft +	+	++	ft +	-
240	+++	+	+	ft +	++	ft +	-

1 cc. yeast suspension (1 g. yeast + 1 g. sucrose in 10 cc.  $\text{H}_2\text{O}$ ) + 1 cc. 0.1  $N$  acid.

Control, 1 cc. yeast suspension + 1 cc. water.

In contrast to the inorganic acids the bases  $\text{NaOH}$ ,  $\text{KOH}$ ,  $\text{NH}_3$  may, in 0.05  $N$  strength, in the presence of sugar, cause only very slight or no liberation of substances giving the nitroprusside reaction within 3 hours (Table VII). One might suspect that this was due to the conversion of GSH liberated first of all into  $\text{G}_2\text{S}_2$  during the period of incubation in alkaline solution. That this is not the reason is shown by the following considerations: (1) if the test be made within 1 or 2 minutes only of the addition of alkali the result is likewise negative; (2) if yeast were very sensitive to the action of alkalis in this respect the nitroprusside reaction would never be negative, for the strength of  $\text{NH}_3$  reached in the colour test as carried out in these experiments is from 1.2 to 2.5  $N$ ; (3) greater concentrations of alkali (0.1  $N$   $\text{NaOH}$  or  $\text{KOH}$  without sugar, or half-saturated  $\text{Na}_2\text{CO}_3$  or  $\text{K}_2\text{CO}_3$ ), which might be expected to promote still more the oxidation of glutathione, can produce strong positive reactions (Table VII). With 0.1  $N$  solutions  $\text{NaOH}$  is generally more active than  $\text{KOH}$ , and  $\text{KOH}$  more active than  $\text{NH}_3$ .

Perhaps the reason for the great variability of the results obtained with the weaker acid and alkaline solutions is that these affect the interchanges between GSH and  $\text{G}_2\text{S}_2$ , which are dependent upon the conditions for oxidation, which again are dependent upon the particular method by which the experiment is carried out (large or small quantity of suspension in narrow or wide vessels, frequency of stirring).

Table VII.

Time (mins.)	NaOH 0.05 N	NH <sub>3</sub>				
		3 N	0.5 N	0.1 N	0.05 N	0.01 N
15	ft +	ft +	ft +	—	—	—
35	ft +	ft +	ft +	—	—	—
60	—	++	+	ft +	ft +	—
120	—	+++	++	+	ft +	ft +
180	—	+++	+++	+	ft +	ft +
1 g. yeast + 1 g. sucrose in 20 cc. alkali solution.						
NaHCO <sub>3</sub>	A	—		KHCO <sub>3</sub>	A	—
	B	—			B	—
Na <sub>2</sub> CO <sub>3</sub>	A	+++		K <sub>2</sub> CO <sub>3</sub>	A	+++
	B	—			B	—
(NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub>	A	++		Control		—

Results after 60 minutes' incubation.

A = saturated solution.

B = 1.25 % solution. This concentration was tested as a control on experiments with alkali salts of bile acids described below.

1 cc. yeast suspension (1 g. yeast + 1 g. sucrose in 10 cc. H<sub>2</sub>O) + 1 cc. A or B.

#### ORGANIC COMPOUNDS.

Table VIII represents an attempt to summarise the results which have been obtained with various organic compounds. The + signs are intended to represent the maximum nitroprusside reaction usually observed in a series of tests carried out at various times during 3 to 5 hours' incubation. The table shows that the sodium salts of the mixed bile acids, and of cholic and deoxycholic acids, have a powerful action even in concentrations as low in some cases as 0.04 %. Many compounds of the class of phenols (phenol, *p*-cresol, thymol, salicylic acid and its two isomers, hexylresorcinolcarboxylic acid,  $\alpha$ - and  $\beta$ -naphthol) and aniline, and the naphthylamines and their tetrahydro-derivatives are active in concentrations from 0.1 to 0.01 *M*. Hexylresorcinolcarboxylic acid is of interest on account of its solvent action upon Gram-negative micro-organisms [Bleyer, 1927]. Of the alcohols tested only caprylic alcohol (saturated solution in water filtered from excess of alcohol) gave a satisfactory positive result. Ethyl alcohol has an increasing inhibitory effect upon the nitroprusside reaction in concentrations from 20 vols. % onwards, until at 50 vols. % the colour reaction (of yeast in thymol-water or salicylic acid solution) is inhibited almost completely; but amounts of this alcohol which might occur in sugar solutions (10 to 15 vols. %) have no perceptible effect upon the colour. Methyl alcohol in concentrations of 1 to 50 vols. % produces weak positive reactions, but can be shown to have at the same time an inhibitory effect; and glycerol (25 vols. %) and formaldehyde (5 to 10 vols. %) suppress the colour test; these three compounds are therefore excluded from the table as possible false negatives. Acetaldehyde (3 vols. %, about 0.5 *M*) gives a negative result, and in this concentration has only a very slight inhibitory effect; while the trichloro-derivative, chloral hydrate, gives a positive result in much weaker solutions (0.066 *M*; cf. the relative potency of trichloroacetic acid and acetic acid, under "Acids" above).

Table VIII.

	Concentration		
	%	Molar approx.	
Na cholate ... ..	4.0	—	4 +
Na deoxycholate ... ..	2.0	—	4 +
Na salts, mixed bile acids, ox bile	2.0	—	4 +
Hexylresorcinolcarboxylic acid ...	0.6 as Na salt	0.025	4 +
Phenol ... ..	1.0-0.1	0.1-0.01	} see Table XII
Salicylic acid ... ..	0.2	0.014	
<i>m</i> - and <i>p</i> -Hydroxybenzoic acid ...	0.2	0.014	
<i>p</i> -Cresol ... ..	0.5	0.05	5 +
Thymol saturated ... ..	0.07	0.005	2 + to 6 +
$\alpha$ -Naphthol ... ..	1.44 as Na compound	0.1	++
$\beta$ -Naphthol ... ..			
Aniline ... ..	1.0	0.1	2 + to 4 +
Caprylic alcohol saturated ...	?	—	++
Inositol ... ..	5.0	0.3	—
Acetaldehyde ... ..	—	0.5	—
Chloral hydrate ... ..	1.0	0.066	++
Chloroform saturated ... ..	—	—	+ to 4 +
Ether saturated ... ..	—	—	++
Ethyl acetate ... ..	6.0	0.7	3 +
Urea saturated ... ..	—	—	4 +
Urea half-saturated ... ..	—	—	+
Urethane ... ..	1.8	0.2	++
Na oleate ... ..	1.0-0.1	0.03-0.003	} see Table XIII
Na stearate ... ..	0.5	0.015	
Quinine-HCl ... ..	3.0	—	
Saponin ... ..	1.0	—	++
KCN ... ..	—	0.1	—

1 g. yeast in 10 cc. watery solution. No sugar.

A test with saturated urea was suggested by Ramsden's [1902] discovery of the macerating action of this solution; the result was strongly positive and even the half-saturated solution is active with some yeasts. The allied compound urethane gives a positive result in much lower concentration (0.2 *M*) and perhaps for quite different reasons.

There is a curious difference between the actions of naphthalene and of its half-reduced form tetrahydronaphthalene; after 24 hours the former gives a strong positive, the latter a negative result. This is shown in Table IX together with the effects of three other naphthalene derivatives. Sodium oleate appears to be about 20 times more active than sodium stearate.

Salicylic acid and phenol are of interest on account of the very low concentrations in which they are active (Table XII). Salicylic acid (0.2 %), which is a nearly saturated solution, is very effective, and with some yeasts one-tenth of this concentration, or 0.0014 *M*, produces a distinctly stronger nitroprusside reaction than that seen in a control. We have made a number of experiments with the three isomers, salicylic acid and *m*- and *p*-hydroxybenzoic acids; the usual result, at 0.2 % strength, is that the *o*-acid is more active than the *p*-, and this again more active than the *m*-acid; evidence was obtained that at a lower concentration (0.1 %) this relation is altered, so that salicylic acid becomes the weakest of the three; but there were some irregularities and this interesting point requires further experiments. From the pharmacological inertness of the *p*- and *m*-compounds one might expect a very

distinct difference. Lafar [1906] states that, at 0.1 % strength, salicylic acid arrests the multiplication of yeast, while the two isomers have no effect. Sodium salicylate, though very effectual in saturated solution (Table II), is less active in dilute solution (0.2 %) than the free acid.

Table IX.

Time	$\alpha$ -Naphthyl- amine-HCl	Naphthalene	Tetrahydro- naphthalene	Tetrahydronaphthylamine		Control in gum
				Ar- $\alpha$	Ac- $\beta$	
0 min.	+++	-	-	ft+	+++	-
60 mins.	+++	ft+	-	+++	+	-
120 "	+++	$\pm$	-	+++	+	-
210 "	+++	-	-	+++	ft+	$\pm$
270 "	+++	-	-	+++	-	ft+
23 hrs.	Solution too dark for test		-	ft+	-	ft+

*Solutions.*  $\alpha$ -Naphthylamine 1.43 g. + 10 cc. *N* HCl  $\rightarrow$  100 cc. = 0.1 *M*.  
 Naphthalene 0.13 g. in 2.6 cc. alcohol + 7.4 cc. gum = 0.1 *M*  
 Tetrahydronaphthalene 1 cc. + 10 cc. gum = approx.  $\frac{2}{3}$  *M*.  
 Tetrahydronaphthylamine 0.15 g. in 10 cc. gum = 0.1 *M*.  
 1 g. yeast suspended in 10 cc. of each solution.  
 Control 1 g. yeast in 10 cc. gum.  
 Gum = 5 % gum-acacia in water.

A result of the same type is obtained when the naphthalene and tetrahydronaphthalene are in the same molar concentrations.

Table X.

Time	In H <sub>2</sub> O			In H <sub>2</sub> O + 10 % sucrose		
	Control	KCN		Control	KCN	
		0.1 <i>M</i>	0.01 <i>M</i>		0.1 <i>M</i>	0.01 <i>M</i>
0 min.	$\frac{1}{2}$ +	-	$\frac{1}{2}$ +	-	$\frac{1}{2}$	-
60 mins.	+	-	-	-	-	-
120 "	+++	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	-
180 "	++	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	-	-
240 "	+++	+	$\frac{1}{2}$ +	+	-	ft+
300 "	++	$\frac{1}{2}$ +	$\frac{1}{2}$ +	$\frac{1}{2}$ +	-	ft+
28 hrs.	+++	++	+++	+++	+	+++

The effect of potassium cyanide is to be seen in Table X; this compound causes a considerable lessening of the natural autolytic reaction of the yeast. It so happened that the yeast used in this experiment gave by itself, in the absence of sugar, an exceptionally strong (up to 3+) colour, hence the inhibitory action of the cyanide was well shown. Voegtlin, Johnson and Dyer [1926] find that glutathione, cystine and cysteine act as antidotes to NaCN, which changes the first two of these three compounds to the reduced forms.

#### INTRACELLULAR AND EXTRACELLULAR LIBERATION.

When the positive results recorded above are considered, the question arises whether the reducing substances liberated are retained within the yeast cell, or pass out into the surrounding fluid. If a series of suspensions of yeast in different media be prepared and incubated and the nitroprusside test be

applied to each emulsion and to the clear fluid obtained from it by centrifuging, some remarkable differences are observed; under the action of some reagents the reducing substances are confined to the cells, the surrounding fluid giving no colour at all with nitroprusside, whilst with other reagents the clear fluid, and the whole emulsion, show colours of equal intensity. Thus a suspension of yeast in 0.2 % salicylic acid will give after from 1 to 4 hours' incubation a very strong (3 +) reaction, while the clear supernatant fluid gives no colour whatever; on the other hand a suspension in 1 or 2 % aniline or phenol will show the same depth of tint in the whole emulsion and in the centrifuged fluid. Thus some reagents cause chiefly an intracellular, and some also an extracellular, liberation of substances giving the nitroprusside reaction. No claim is made here that these distinctions are absolute; the differences are those of degree, and the important factor is of course the duration of exposure to the reagent. When incubation is continued for many hours extravasation from the cells increases and positive tests begin to be obtained from fluids previously negative; yet even after 24 hours' incubation the contrasts may still be very distinct. Different samples of yeast may give more or less variable results. With some agents (inorganic acids, saturated urea solution, chloroform and thymol water) which produce an effect of the intracellular (A) type there is an especial tendency to change to the B type as incubation is continued. Table XI summarises the range of results obtained in over 800 colour tests, on about 25 samples of yeast.

Table XI.

A. Intracellular type	B. Extracellular type
Phenol 0.5 % or less Salicylic acid 0.2 % <i>m</i> - and <i>p</i> -Hydroxybenzoic acid Na cholate Na deoxycholate Na oleate 1.0 to 0.02 % Caprylic alcohol saturated in H <sub>2</sub> O Chloroform saturated in H <sub>2</sub> O Ether saturated in H <sub>2</sub> O Thymol saturated in H <sub>2</sub> O Chloral hydrate 1 % Urea saturated or half-saturated in H <sub>2</sub> O Urethane 0.2 <i>M</i> Saponin 1 % KNO <sub>3</sub> saturated HNO <sub>3</sub> , H <sub>2</sub> SO <sub>4</sub> , HCl, 0.1 <i>N</i> (at first) Autolysis	Phenol 1 % Aniline 2 % β-Naphthol 0.1 <i>N</i> as Na compound α-Naphthylamine-HCl 0.1 <i>N</i> Hexylresorcinolcarboxylic acid, 1.25 % Na salt Saturated Na benzoate Quinine-HCl 3 % Ethyl acetate 6 % Trichloroacetic acid 10 % to 0.8 % (0.05 <i>M</i> ) CaCl <sub>2</sub> , 1 <i>M</i> , 0.75 <i>M</i> , 0.5 <i>M</i> NaCl 6 <i>M</i> NH <sub>4</sub> Cl saturated Na arsenite 1.5 <i>M</i> Heat (100°, 65°) Grinding Freezing

1 g. yeast in 10 cc. watery solution. No sugar. Tests made usually after 30 minutes' to 5 hours' incubation.

The demonstration of the nitroprusside reaction in the cells separated from the fluid was not attempted since this would require a process of washing and centrifuging which might cause secondary changes. The whiteness of the yeast lessens the pink colour in the whole suspension, and some allowance must be made for this in recording comparisons with the supernatant fluid.



Table XII.

		Hours							
		1.5		3		4.5		23	
		F	E	F	E	F	E	F	E
Phenol	1 %	4+	4+	4+	4+	4+	4+	ft+	½+
	0.5	-	+	-	½+	-	ft+	4+	3+
	0.2	-	++	-	++	-	+	-	3+
	0.1	-	+	-	+	-	+	-	3+
Salicylic acid	0.2 %	-	3+	-	3+	?	4+	++	4+
Control in H <sub>2</sub> O		-	ft+	-	ft+	-	ft+	-	++

F=supernatant fluid after centrifuging.

E=whole emulsion.

1 g. yeast in 10 cc. solution or H<sub>2</sub>O. No sugar.

Table XII represents a series of changes which has been observed with no great variations in each one of six samples of yeast tested. The figures show (1) that phenol in 1 % concentration produces the extracellular, and salicylic acid in very nearly saturated solution the intracellular, type of change; (2) that phenol in 0.5 % or weaker solutions produces the intracellular liberation only; (3) that 0.2 % phenol is more effective than the 0.5 % solution—we have noted other instances where the lesser amount of a reagent produced the greater effect, and some of these are under investigation; (4) that after a whole day's incubation the change, at first intracellular, may become extracellular (0.5 % phenol, and salicylic acid); (5) that the reducing substances formed in autolysis (control in H<sub>2</sub>O) are not detected outside the cell; this has been the case in all of 16 controls examined in this way. Table XIII illustrates the intracellular type of action of sodium oleate, and its activity in high dilutions, 0.1 % (0.003 *M*) or less.

Table XIII.

		30 mins.		90 mins.		150 mins.		20 hrs.	
		F	E	F	E	F	E	F	E
Sodium oleate	1 %	-	++	ft+	+++	½+	+++	+	++
	0.1	-	½+	-	+++	-	+++	++	++++
	0.02	-	-	-	ft+	-	+	-	+++
Control in H <sub>2</sub> O		-	-	-	-	-	-	-	ft+

F=supernatant fluid of centrifuged emulsion.

E=whole emulsion.

1 g. yeast in 10 cc. solution or H<sub>2</sub>O. No sugar.

## SERA AND ENZYMES.

Yeast treated with an antiserum, prepared by giving six intravenous injections of yeast suspension to a rabbit, gave no nitroprusside reaction, although there was considerable agglutination. Peptic digestion usually gives a positive, and tryptic digestion a negative, result, but the experiments were not controlled fully by corresponding acid and alkaline solutions. Digestion with takadiastase was likewise negative in result.

## MICROSCOPICAL EXAMINATION.

The microscopical examination of yeast exposed to the various physical and chemical agents enumerated here has given almost wholly negative results (the changes brought about by grinding are described separately above). Fragmentation of cells, and cell débris, such as are seen in abundance when mammalian cells are exposed to some bile salt preparations, were not seen in one single instance. Occasionally some effects were noted (some shrinkage in 10 % trichloroacetic acid and in saturated ammonium chloride; a glassy appearance in saturated sodium benzoate; clumping in a tryptic digest and in anti-yeast serum) but no appearance of cytolysis. The two classes of reagents which seem to produce chiefly intracellular, or extracellular, liberation of reducing substances showed no differences.

## DISCUSSION.

When the results presented in the tables above are considered, the number and great variety of agents which cause yeast to give a positive nitroprusside reaction may suggest that this effect can be brought about by anything which does considerable damage to the cell, and that the findings therefore do not indicate any specific processes and are hence lacking in interest. But when one regards some of the agents which give a negative or only very weak positive result (heating for 2 minutes at 59°; X-rays in the doses stated; 0.1 M KCN; a large number of saturated salt solutions) it is difficult to believe that these are not injurious. Hence there seem to be two classes of harmful factors: those which do, and those which do not, cause a considerable liberation of reducing substances, and this points to a field for further experiments. To decide whether yeast is or is not killed by the various conditions giving the positive and negative results would require a lengthy investigation by methods of subculture which we did not wish to pursue, since we were not interested in the biology of yeast itself so much as in suggestions for work on mammalian cells.

The experiments described above were not capable of showing to what extent substances other than glutathione contribute to the nitroprusside reactions observed; the slight differences in tint of the colour test with some reagents, *e.g.* a reddish tinge with  $\text{CaCl}_2$ , suggest that the processes occurring are not always quite the same in nature. In this connection, one may note that Tunncliffe's method for the estimation of glutathione employs two factors, namely, grinding, and trichloroacetic acid, which are among those found in the present investigation to be most effective, and to cause extracellular liberation. Tunncliffe's [1925] method was checked by parallel estimations of organic sulphur. It would be of some interest to compare the amount of glutathione obtained from a sample of yeast by this method with the amount of nitroprusside-reacting substance liberated by such agents as heat or phenol.

Although no cytolysis was observed microscopically the reactions described here have no doubt some relation to those of haemolysis; some of the most active factors (freezing, bile salts, sodium oleate, saponin, ether, chloroform) are well-known haemolytic agents. It is noteworthy that some substances which have a powerful effect in lowering surface tension (bile salts, sodium oleate, saponin, caprylic alcohol, ether) nevertheless produce the intracellular rather than the extracellular type of liberation.

The results of most interest seem to be those in which the amount of reagent required to cause a liberation of reducing substances is very small. Thus phenol, salicylic acid, thymol and sodium oleate are active in strengths from 0.014 *N* to 0.003 *N*. An experiment with thymol water, such as those which provided the figures given in Table VIII, consists in the addition of about 7 mg. of thymol to 1000 mg. of compressed yeast, and this gives a very strong reaction. Further experiments with these and with still more dilute solutions are in progress.

These experiments were almost completed when we learned of the work of Harris [1923] on the denaturation of proteins; many of his results obtained with egg-albumin, such as the action of heat, of incubation with acids and alkalis, and of ultra-violet light, and the greater apparent effect of peptic as compared with tryptic digestion, are very similar to some of those described here. It is perhaps worth noting that some of the substances (sodium salicylate, sodium oleate, bile) stated by Vincent [1928] to have a remarkable neutralising action upon bacterial toxins are among those which produce a nitroprusside reaction; possibly both phenomena depend upon a process of denaturation.

#### SUMMARY.

A large number of agents have been examined with regard to their power to liberate from yeast substances giving the nitroprusside reaction. Positive results were obtained by a variety of methods, some physical (heating above a certain temperature, freezing, grinding, ultra-violet light), some chemical (*e.g.* exposure to certain saturated salt solutions, or to many organic compounds of the phenol and amine classes in solutions of 0.1 to 0.01 *M* strength). Negative results are more difficult to establish, because the nitroprusside test is inhibited by many compounds (*e.g.* sodium nitrite, ethyl alcohol, toluene); hence an apparent negative result with yeast may be in reality a positive one in which the colour test is masked. The factors which produce from yeast substances giving the nitroprusside test can be separated to a certain extent into two classes; in one class the liberation is chiefly intracellular, in the other it is extracellular also. Many of the positive agents are known to be haemolytic (bile salts, saponin, sodium oleate, freezing) but lysis of the yeast cell was not observed in any instance.

We are indebted to Dr J. O. Harvey of the Electrical and Radio-therapeutic Department of the Cancer Hospital for carrying out the exposures of yeast to radiations.

## REFERENCES.

- Barry, Bunbury and Kennaway (1928). *Biochem. J.* **22**, 1102.  
Bleyer (1927). *Biochem. Z.* **181**, 350.  
Euler and Lindner (1915). *Chemie der Hefe und der alkoholischen Gärung* (Leipzig), p. 301.  
Harris (1923). *Proc. Roy. Soc. Lond. B*, **94**, 426.  
Hurtley (1913). *Lancet*, i, 1160.  
Lafar (1906). *Handbuch der technischen Mykologie* (Jena), Bd. iv, p. 139.  
Ramsden (1902). *J. Physiol.* **28**, *Proc. Physiol. Soc.* xxiii.  
Tunnicliffe (1925). *Biochem. J.* **19**, 194.  
Vincent (1928). *Compt. Rend. Acad. Sci.* **186**, 1175.  
Voegtlin, Dyer and Leonard (1923). *Pub. Health Rep. U.S. Pub. Health Service*, **38**, 1882.  
——— (1925). *J. Pharm. Exp. Ther.* **25**, 297.  
Voegtlin, Johnson and Dyer (1926). *J. Pharm. Exp. Ther.* **27**, 467.

# CXXXVIII. THE EFFECT OF FLUORIDES AND IODIDES ON THE CLOTTING OF MILK BY PEPSIN.

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*(Received July 10th, 1928.)*

SOME effects of halogen salts on the clotting of milk by pepsin have been described in a previous paper [Clifford, 1927]. With the fluorides and iodides of Na, K, and  $\text{NH}_4$  inhibitory actions were found which varied with the concentration of salt used. It was therefore thought advisable to continue the research with a view to locating more exactly the molar concentration at which the inhibitory influence reached its maximum.

In the experiments published earlier, results were obtained of which the following with NaF is typical:

Molar concentration 0.035. Coagulation time 1 min. 43 secs.

„ 0.0175. „ no clot formed in 6 hrs.

Similar changes occurred with K and  $\text{NH}_4$  fluorides and with Li, Na, K and  $\text{NH}_4$  iodides. These results are in agreement with those of McMeekin [1928], who, using egg-white and pepsin, finds Na and K iodides to exert an inhibitory effect when added in a concentration greater than  $N/256$ . But, as previously shown [Clifford, 1927], the writer cannot agree that a similar inhibitory effect is shown by the bromides and chlorides of these metals, which actually hasten coagulation up to a concentration of 0.14–0.28 *M*. The differences may be due to the fact that, in one case the reaction is a hydrolysis, and in the other an aggregation of a colloidal system.

In this paper, experiments are described using halogen salt solutions of strengths intermediate between that causing very little, if any, alteration in the rate of coagulation of milk by pepsin, and that which caused complete inhibition of clotting for a period of  $2\frac{1}{2}$ – $6\frac{1}{2}$  hours.

## EXPERIMENTAL METHOD.

The method of determining the end-point of the reaction (*i.e.* the first formation of a clot) has been described in a previous paper [Clifford, 1927]. As before, a solution of commercial pepsin scales was used which gave a coagulation time of  $1\frac{1}{2}$ –3 minutes when 2 cc. of it were added to a mixture of 10 cc. milk and 2 cc. distilled water, all three solutions having first been brought to a temperature of  $37^\circ$ .

A series of 21 test-tubes was then taken, and into each tube 10 cc. milk were pipetted. Then

	to tube (1)	2 cc.	dist. water and	0.0 cc.	halide
	"	(2)	1.9 cc.	"	0.1 cc. "
	"	(3)	1.8 cc.	"	0.2 cc. "
and	"	(21)	0.0 cc.	"	2.0 cc. "

were added.

The concentration of the salt solution used was obtained from the results of the experiments published earlier [Clifford, 1927]. It was the lowest concentration which was first found completely to inhibit coagulation when 2 cc. of it were added to 10 cc. milk and 2 cc. of pepsin. In every case the experiments were repeated with two separate batches of commercial pepsin scales, and at least two samples of each halide.

## RESULTS.

Sodium fluoride. Concentration of solution added: 0.5 *M*.

Tube no.	cc. of 0.5 <i>M</i> NaF in 14 cc. mixture	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	2	35	0.0000
(2)	0.1	1	59	0.0038
(3)	0.2	1	51	0.0072
(4)	0.3	2	52	0.0108
(5)	0.4	5	0	0.0144
(6)	0.5	—		0.0180
(7)	0.6	No clot in 6½ hours		
(21)	2.0	No clot in 6½ hours		

Potassium fluoride. Concentration of solution added: 0.25 *M*.

Tube no.	cc. of 0.25 <i>M</i> KF	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	1	16	0.0000
(2)	0.1	1	11	0.0018
(3)	0.2	1	11	0.0036
(4)	0.3	1	12	0.0054
(5)	0.4	1	21	0.0072
(6)	0.5	1	30	0.0090
(7)	0.6	1	46	0.0108
(8)	0.7	2	12	0.0126
(9)	0.8	2	6	0.0144
(10)	0.9	2	15	0.0162
(11)	1.0	2	15	0.0180
(12)	1.1	2	28	0.0198
(13)	1.2	No clot in 3½ hours		0.0216
(21)	2.0	No clot in 3½ hours		

Ammonium fluoride. Concentration of solution added: 0.25 *M*.

Tube no.	cc. of 0.25 <i>M</i> NH <sub>4</sub> F	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	0	53	0.0000
(2)	0.1	0	55	0.0018
(3)	0.2	0	54	0.0036
(4)	0.3	1	12	0.0054
(5)	0.4	1	15	0.0072
(6)	0.5	2	4	0.0090
(7)	0.6	2	34	0.0108
(8)	0.7	2	51	0.0126
(9)	0.8	4	57	0.0144
(10)	0.9	11	31	0.0162
(11)	1.0	13	13	0.0180
(12)	1.1	No clot in 4½ hours		0.0198
(21)	2.0	No clot in 4½ hours		

Lithium iodide. Concentration of solution added: 0.25 *M*.

Tube no.	cc. 0.25 <i>M</i> LiI	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	2	30	0.0000
(2)	0.1	2	27	0.0018
(3)	0.2	2	13	0.0036
(4)	0.3	2	26	0.0054
(5)	0.4	2	48	0.0072
(6)	0.5	3	4	0.0090
(7)	0.6	4	0	0.0108
(8)	0.7	4	7	0.0126
(9)	0.8	4	53	0.0144
(10)	0.9	5	4	0.0162
(11)	1.0	5	0	0.0180
(12)	1.1	8	7	0.0198
(13)	1.2	No clot in 4½ hours		0.0216
(21)	2.0	No clot in 4½ hours		

Sodium iodide. Concentration of solution added: 0.5 *M*.

Tube no.	cc. 0.5 <i>M</i> NaI	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	1	42	0.0000
(2)	0.1	1	37	0.0036
(3)	0.2	1	41	0.0072
(4)	0.3	1	43	0.0108
(5)	0.4	2	0	0.0144
(6)	0.5	2	13	0.0180
(7)	0.6	2	21	0.0216
(8)	0.7	2	33	0.0252
(9)	0.8	2	43	0.0288
(10)	0.9	3	0	0.0322
(11)	1.0	No clot in 3½ hours		0.0360
(21)	2.0	No clot in 3½ hours		

Potassium iodide. Concentration of solution added: 0.5 *M*.

Tube no.	cc. 0.5 <i>M</i> KI	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	1	25	0.0000
(2)	0.1	1	28	0.0036
(3)	0.2	1	27	0.0072
(4)	0.3	1	38	0.0108
(5)	0.4	1	47	0.0144
(6)	0.5	1	52	0.0180
(7)	0.6	2	2	0.0216
(8)	0.7	2	15	0.0242
(9)	0.8	2	21	0.0288
(10)	0.9	2	25	0.0324
(11)	1.0	3	8	0.0360
(12)	1.1	No clot in 3½ hours		0.0396
(21)	2.0	No clot in 3½ hours		

Ammonium iodide. Concentration of solution added: 2 *M*.

Tube no.	cc. 2 <i>M</i> NH <sub>4</sub> I	Coagulation time		Molar concentration
		m.	s.	
(1)	0.0	2	5	0.0000
(2)	0.1	1	27	0.0143
(3)	0.2	1	19	0.0286
(4)	0.3	1	23	0.0429
(5)	0.4	1	45	0.0562
(6)	0.5	2	0	0.0715
(7)	0.6	2	30	0.0848
(8)	0.7	3	7	0.1001
(9)	0.8	3	25	0.1144
(10)	0.9	4	12	0.1287
(11)	1.0	4	37	0.1430
(12)	1.1	4	45	0.1573
(13)	1.2	6	17	0.1716
(14)	1.3	10	22	0.1859
(15)	1.4	13	47	0.2002
(16)	1.5	No clot in 4½ hours		0.2145
(21)	2.0	No clot in 4½ hours		

The outstanding feature, as can be readily seen from Fig. 1, is the sudden change from a coagulation time of, at the utmost, 14 minutes, and generally 3–5 minutes, to a completely inhibitory effect, with a very small alteration in concentration of salt in the mixture, amounting to an increase of 0.0036 *M* only.

The order of magnitude of the change in concentration and also the actual low molarity of salt in the experimental mixture (*e.g.* 0.018 *M* NaF) seems to preclude the idea that the results are a consequence of a change in hydrogen ion concentration, especially in a well buffered system such as milk.

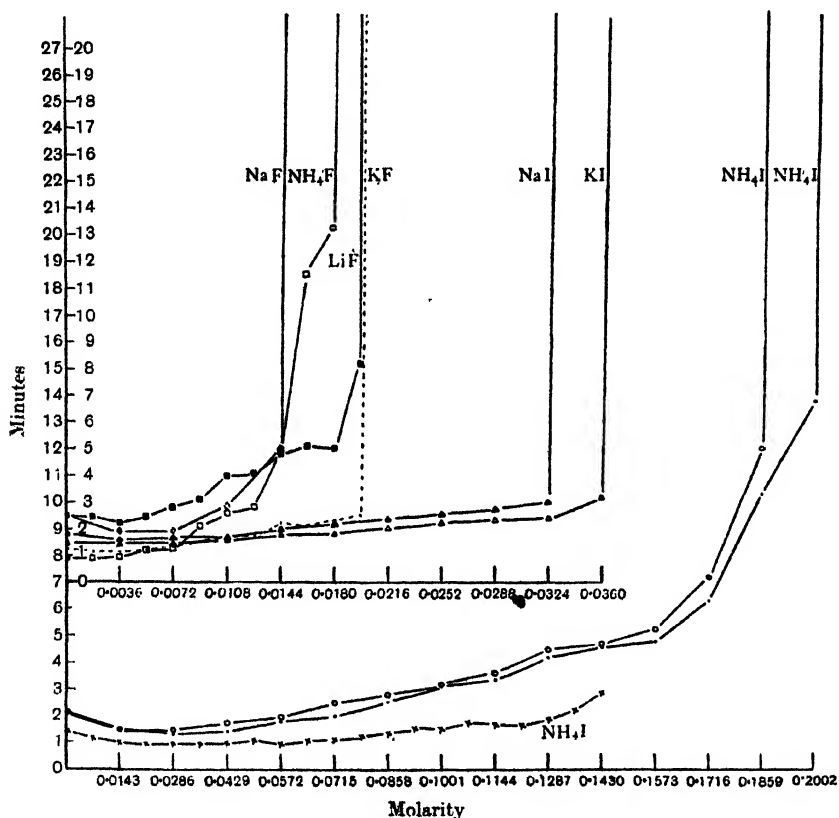


Fig. 1.

There is a marked difference between the behaviour of NH<sub>4</sub>I as opposed to the iodides of Li, Na and K since the molar concentration for inhibition has to be about ten times greater, but the shape of the curve obtained is the same as for the other iodides. Taken together with the fact that NH<sub>4</sub>F showed a rather less sudden change from coagulation to inhibition of reaction than did either NaF or KF, it appears that the NH<sub>4</sub> radicle favours clotting, though the halide radicle is prepotent.



Curves from three experiments with  $\text{NH}_4\text{I}$  using two different samples of the salt are given. Several others have been obtained, and all fall within the limits of these curves.

#### SUMMARY.

(1) The clotting of milk by pepsin is inhibited by the addition of the fluorides of Na, K and  $\text{NH}_4$  and the iodides of Li, Na, K and  $\text{NH}_4$ .

(2) This inhibitory action depends on the concentration of the halide.

(3) The inhibition of coagulation occurs suddenly at a definite molarity of the halide added, a difference of 0.0036  $M$ , changing the rate from 5–13 minutes to absence of change for 4–6 hours.

(4) The inhibitory action of fluorides is greater than that of iodides.

(5) The concentration of  $\text{NH}_4\text{I}$  has to be six times that of  $\text{NaI}$  or  $\text{KI}$ , and ten times that of  $\text{LiI}$  to produce a similar inhibitory effect.

The expenses of this research were defrayed by a grant from the Medical Research Council.

#### REFERENCES.

- Clifford (1927). *Biochem. J.* **21**, 544.  
McMeekin (1928). *J. Biol. Chem.* **78**, xliii.

## CXXXIX. THE COLOUR REACTIONS OF SUBSTANCES CONTAINING VITAMIN D.

By WILFRED ARCHIBALD SEXTON.

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*(Received May 22nd, 1928.)*

SHEAR and KRAMER [1926] have found that on boiling oils containing the antirachitic vitamin with a solution of aniline hydrochloride in excess of aniline a characteristic red colour is produced, and suggest that this reaction may be employed in the detection of vitamin D. The empirical nature of such a test casts some doubt on its reliability as a sure method of detecting the vitamin, especially as it has recently been demonstrated that two biologically inactive substances, ergosterol peroxide and ergopinacone, give the blue colour with antimony trichloride which is a characteristic property of vitamin A [Windaus, Borgeaud and Brunken, 1927].

With a view therefore to investigating the specificity of this reaction, the effect of the reagent upon a number of sterol derivatives has been studied. The aniline hydrochloride solution, which was almost colourless, was prepared exactly as described by Shear and Kramer by mixing five parts of redistilled aniline with one part by volume of concentrated hydrochloric acid. Cod-liver oil was found to respond to the test exactly as described by these authors. On shaking together the oil and the reagent a green emulsion was first formed. Heat soon destroyed the green colour and after boiling for half a minute and allowing the two layers to separate, the lower layer appeared intensely red. In the case of "essogen," a highly concentrated preparation of vitamins A and D, for a sample of which I am indebted to Messrs Lever Bros., Ltd., the initial green colour was not noted, though on heating the oil behaved in a similar manner to cod-liver oil.

In the case of the solid substances, about 0.05 g. was introduced into a test-tube and 3 cc. of the aniline preparation were added. The liquid was heated to boiling and boiled for 30 seconds. The results are indicated in the table. A red colour was produced by irradiated ergosterol, while the unirradiated sterol caused only a slight darkening of the liquid. The hydrocarbons were without effect as also were cholesterol, cholesteryl acetate and dicholesteryl ether. The compounds which proved most sensitive were the ketones, and of these the singly unsaturated ketones alone gave the red colour to any marked degree. The saturated ketones brought about slight darkening, while the doubly unsaturated oxycholesterylene gave a deep brown. For the purpose of comparison two terpene ketones, camphor and carvone, were examined. These

behaved in a similar manner to their sterol analogues, the saturated camphor causing slight darkening while the doubly unsaturated carvone produced a brown colour.

These results show clearly that the test is by no means specific for vitamin D. On the other hand, the positive reaction given by irradiated ergosterol and by the ketones is in harmony with the view expressed by Heilbron, Morton and Sexton [1928] that the antirachitic vitamin is possibly ketonic in character. It must be borne in mind however that the photochemical formation of vitamin D from ergosterol is by no means a quantitative process. It is not impossible therefore that the ketone which may be produced during the irradiation of ergosterol is not the vitamin.

Bezssonoff [1924] has described a preparation of phosphomolybdotungstic acid which gives a colour with oils which contain the antirachitic vitamin. Cod-liver oil and "essogen" were found to respond to the test as described by Bezssonoff, but irradiated ergosterol gave no colour at all. The colour produced by the oils must therefore be due to some constituent other than vitamin D.

Substance	Type	Colour
Cholestane	Hydrocarbon, saturated	Nil
Cholestene	Hydrocarbon, 1 double bond	Nil
$\psi$ -Cholestene	Hydrocarbon, 1 double bond	Nil
Cholesterylene	Hydrocarbon, 2 double bonds	Slight darkening
Cholesterol	Alcohol, 1 double bond	Nil
Cholesteryl acetate	Ester, 1 double bond	Nil
Dicholesteryl ether	Ether, 2 double bonds	Nil
Ergosterol	Alcohol, 3 double bonds	Slight darkening
Irradiated ergosterol	—	Reddish brown which becomes deeper and redder after 20 minutes
Cholestane-4-one	Saturated ketone	Slight darkening. Prolonged standing (24 hours) in air gives red
Cholestane-7-one	Saturated ketone	Slight darkening. Prolonged standing (24 hours) in air gives red
Cholestenone	Ketone, 1 double bond	Orange red, becoming blood red in 10 minutes
$\beta$ -Oxycholestenol acetate	Ketone, 1 double bond	Orange red, becoming blood red in 10 minutes
Oxycholestenone	Diketone, 1 double bond	Blood red, rapidly deepening
Camphor	Saturated ketone	Slight darkening
Oxycholesterylene	Ketone, 2 double bonds	Brown, darkening on standing
Carvone	Ketone, 2 double bonds	Brown, darkening on standing

#### REFERENCES.

- Bezssonoff (1924). *Compt. Rend. Acad. Sci.* **179**, 572.  
 Heilbron, Morton and Sexton (1928). *J. Chem. Soc.* 47.  
 Shear and Kramer (1926). *Proc. Soc. Exp. Biol. Med.* **23**, 546.  
 Windaus, Borgeaud and Brunken (1927). *Nachr. Ges. Wiss. Göttingen.*

# CXL. CHARACTERISATION OF VERY SMALL QUANTITIES OF PROTEINS BY VAN SLYKE'S METHOD.

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AND MOTHNAHALLI SREENIVASAYA.

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*(Received May 25th, 1928.)*

IN the course of our studies on animal and vegetable proteins, it became necessary to deal with very small quantities of substances, about 100 to 150 mg., a twentieth of the quantity usually employed for a macro-analysis. Thimann [1926] has described a method for the determination of the Hausmann numbers in small quantities of proteins and we have extended the method for a more comprehensive characterisation of proteins by Van Slyke's method.

## EXPERIMENTAL.

*Digestion.* 100 to 150 mg. of the protein were weighed out into a small Kjeldahl flask ( $4 \times 30$  cm.) fitted with a ground glass air-condenser and digested for 36 hours with 10 cc. of 20 % hydrochloric acid. The acid was then removed by distillation *in vacuo* (20–25 mm.) at  $40-45^{\circ}$ . Soda-lime was placed in the receiver and a slow current of ammonia-free air was passed during the distillation to facilitate removal of the acid. A fresh quantity of ammonia-free water was added and the distillation repeated under similar conditions. The titratable acidity of the digest after distillation in the successive duplicates lay between very narrow limits. This fact is of great importance in connection with the subsequent precipitation of the hexone bases, which has been shown by Knaggs [1923] to depend on the concentration of the salts present in the digest of the amino-acids.

The syrupy mass was dissolved in ammonia-free water and made up to about 40 cc. Two portions of 2 cc. each were taken for total nitrogen, two of 10 cc. each for the series of analyses and two of 5 cc. each for the estimation of arginine.

*Amide nitrogen.* Each of the 10 cc. portions was neutralised with  $N/2$  alkali till just pink to phenolphthalein and, after adding a drop of the alkali in excess, the mixture was aerated for 2 hours at room temperature, air free from ammonia and carbon dioxide being passed. A few drops of amyl alcohol were added to prevent foaming. The ammonia was absorbed in 10 cc. of  $N/70$  alkali.

*Melanin nitrogen.* The solution, after aeration, was carefully neutralised with  $N/5$  hydrochloric acid and the melanin filtered through a small Jena glass filter covered with a thin layer of asbestos. The nitrogen in the precipitate was determined by the micro-Kjeldahl process.

*Hexone bases.* The filtrate was evaporated on a water-bath to about 5 cc., washed into a 20 cc. stoppered centrifuge tube and made up to nearly 10 cc. About 1 cc. of concentrated hydrochloric acid was then added. The tube was plugged with cotton wool and autoclaved for 30 mins. at 25 lbs. pressure [Knaggs, 1923]. 3 cc. of 25 % phosphotungstic acid solution were then added and the tube was placed in a boiling water-bath until the precipitate had almost redissolved. After cooling, the tube was placed in the ice-chest for 36 hours. The precipitate was then centrifuged, and washed in the centrifuge thrice with ice-cold hydrochloric acid (1-10), using 3 cc. each time. The clear centrifugate and the washings were passed through a small filter to recover any suspended particles. The combined precipitates were dissolved by the gradual addition of  $N/2$  sodium hydroxide until the pink colour formed with phenolphthalein remained just permanent. The solution was made up to 25 cc. and 5 cc. were employed for total nitrogen and another 5 cc. for amino-nitrogen and 10 cc. for a micro-estimation of sulphur according to Pregl.

From the sulphur content the amount of cystine in the precipitate of the bases was calculated.

*Arginine.* Arginine was estimated by arginase which was prepared from the liver of a ram. The enzyme preparation was found to be free from aminases and amidases which liberate ammonia from amino-acids and amides and its activity on a solution of arginine was determined. 97 to 98 % of the arginine was found to be hydrolysed under the conditions which obtain in the accompanying experiments.

5 cc. portions of the original amino-acid digest were neutralised with  $N/2$  sodium hydroxide, ammonia was removed by aeration as before and the  $p_H$  adjusted to 9.7 by adding a phosphate buffer. After addition of 0.5 cc. of the enzyme extract and 1 cc. of toluene, the reaction was allowed to proceed for 36 hours at 37°. Controls were run with 5 cc. of water and 0.5 cc. of the fresh enzyme extract. Urease solution, corresponding to half a pellet of Dunning's preparation, was then added to the reaction mixture, the  $p_H$  being readjusted to 7. After 12 hours' reaction at 37° a saturated solution of potassium carbonate was added in slight excess and the ammonia estimated by aeration.

From a knowledge of the content of cystine, arginine and the non-amino-nitrogen in the basic fraction, the histidine and lysine contents were calculated.

*Mono-amino-fraction.* The combined filtrates from the precipitation of the hexone bases were made up to 50 cc., of which two 10 cc. portions were used for total nitrogen and two 5 cc. portions for amino-nitrogen.

All ammonia distillations were carried out in the Parnas-Wagner modification of Pregl's micro-apparatus, and the amino-nitrogen determinations made with Van Slyke's micro-apparatus.

Table I. *Caseinogen.*

Results expressed as percentages of total nitrogen.

	Micro Narayana and Sreenivasaya	Macro Narayana and Sreenivasaya	Hoffman and Gortner [1924]	Dunn and Lewis [1921]	Van Slyke [1914]
Ammonia-N	9.90	10.12	10.20	10.49	10.27
Humin-N	1.16	1.86	1.51	2.13	1.28
Basic N:					
Arginine-N	9.89	9.01	9.20	7.42	7.41
Histidine-N	3.88	4.16	6.26	6.01	6.21
Cystine-N	0.96	0.68	1.05	0.48	0.21
Lysine-N	7.67	8.12	8.49	9.09	10.30
N in filtrate from bases:					
Amino-N	59.20	57.30	54.12	58.78	55.81
Non-amino-N	9.02	9.10	8.76	5.93	7.13
Total	101.68	100.35	99.59	100.33	98.61

Table II. *Gelatin.*

Results expressed as percentages of total nitrogen.

	Micro Narayana and Sreenivasaya	Macro Narayana and Sreenivasaya	Micro Thimann [1926]
Ammonia-N	1.84	1.70	2.60
Humin-N	Trace	0.56	0.00
Basic N	22.61	25.04	17.60
Arginine-N	15.87	—	—
Histidine-N	2.74	—	—
Lysine-N	4.00	—	—
N in filtrate from bases	76.2	71.06	79.60
Amino-N	57.40	—	—
Non-amino-N	18.80	—	—
Total	100.65	98.36	99.8

## SUMMARY.

A method for the characterisation of very small quantities of proteins by Van Slyke's method has been described.

Our grateful thanks are due to Prof. R. V. Norris for the keen interest he has evinced during the course of this work.

## REFERENCES.

- Dunn and Lewis (1921). *J. Biol. Chem.* **49**, 327.  
 Hoffman and Gortner (1924). *Coll. Symp. Mon.* **2**, 209.  
 Knaggs (1923). *Biochem. J.* **17**, 488.  
 Thimann (1926). *Biochem. J.* **20**, 1190.  
 Van Slyke (1914). *J. Biol. Chem.* **16**, 531.

# CXLI. THE ACTION OF X-RADIATION UPON VITAMIN D IN ACTIVATED ERGOSTEROL.

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*(Received June 15th, 1928.)*

OUR interest in the possibility that X-rays may have an effect upon vitamin D was aroused by the paper of Hopf and Herman [1926] in which they described a pathological condition produced in rats fed on a diet which had been submitted to the action of X-rays. As this condition bore a striking resemblance to experimental rickets, it was decided to re-investigate the problem and to determine whether the result was due to the direct destruction of the vitamin or to the formation of a substance or substances in the irradiated foodstuffs which might either bring about a pathological condition resembling rickets or indirectly destroy the vitamin. The matter could be decided by submitting activated ergosterol [Rosenheim and Webster, 1927] to the action of X-rays and then examining the antirachitic properties of the product by biological methods.

## METHODS.

Rats were used and fed on a modified, No. 3143, McCollum diet consisting of

Whole wheat coarsely ground or crushed	33
Whole maize coarsely ground or crushed	33
Wheat gluten ... ..	15
Gelatin powdered ... ..	15
Calcium carbonate ... ..	3
Sodium chloride ... ..	1

Lard 1 % and marmite 0.1 % by weight of the dry mixture were added, the food was mixed with distilled water and cooked for 5 minutes. The allowance was 10 g. of the dry mixture per rat per day and unlimited supplies of water. The food was stored dry and cooked fresh each day. The animals did very well on the diet, the cooked food being in our opinion an improvement on the dry mixture.

In a preliminary experiment with eight young rats on this modified diet, it was found that rickets resulted in every case by the end of the 4th week without the general health being adversely affected to any appreciable extent.

The following terms are used to designate the different solutions to be tested for antirachitic value:

- PVD (Pro-vitamin D) = Pure unactivated ergosterol.  
VD (Vitamin D) = Ergosterol activated by ultra-violet rays.  
VDX (Vitamin DX) = VD exposed to X-rays.

*Exp. 1.* The pure non-activated ergosterol (kindly supplied by Dr Rosenheim) was dissolved in absolute alcohol to form a 0.1 % solution; 1 cc. of this solution was transferred to a 30 cc. quartz flask which was then filled with nitrogen and closed with a glass stopper lubricated with rubber grease. The flask was half submerged in tap water at 20°, rotated at 180 r.p.m. and irradiated for 1 hour, the burner consuming 316—311 watts (79—76 volts, 4—4.1 amps).

The activated ergosterol was then transferred to a measuring cylinder, the flask washed out with alcohol and the washings added to the solution in the measuring cylinder, diluting the original to 10 cc. (= 0.01 % solution VD). 5 cc. of this solution was allowed to evaporate to dryness in a watch glass which was then covered with tissue paper and exposed to X-rays. The X-ray exposure, which was started 5 hours after the termination of the ultra-violet irradiation, proceeded for 18 minutes at a distance of 20 cm. from a 10 ma. radiator Coolidge tube excited by alternating current at 65 kv. and 5.6 ma. This turned a Sabouraud pastille lying on metal at the distance of the material under exposure to tint B, *i.e.* a dose of 4 PD. After exposure, the sample was redissolved in absolute alcohol to give a 0.01 % solution (VDX). The alcoholic solution was added to pure olive oil to give a dilution of 0.0004 mg. VDX per cc. Similar solutions of PVD and VD were also prepared.

Three samples of olive oil were thus obtained containing respectively pure ergosterol (PVD), activated ergosterol (VD) and activated ergosterol which had received a known dose of X-rays (VDX). 0.25 cc. of oil containing 0.0001 mg. of the substance to be tested was administered daily to each animal by means of a graduated pipette, except on Sundays. The Sunday dose was added to the Saturday and Monday doses. In the first experiment, 36 young rats of mixed stock and of approximately the same age were used, the weight varying from 31 g. to 48 g. and averaging 40 g. They were divided into three groups and were put on the diet 20 days before oil feeding commenced, in order to diminish their store of vitamin D. Group I received the activated ergosterol (VD), Group II received the activated ergosterol plus X-rays (VDX), Group III received the pure ergosterol, pro-vitamin D (PVD).

### RESULTS.

Three days after oil administration had commenced, three animals died, one from each group and, on examination, two showed slight rickets and one no rickets.

Nine animals were killed after 26 days of oil administration; seven after 37 days and thirteen after 43 days. All showed signs of rickets but of varying severity. The degree of rickets was estimated by examining the costo-chondral junctions of the animals. Those with only a slight beading of the junctions were labelled slight, those in addition with a bending of the rib in one plane, moderate, and those with bending in two planes as well as beading were labelled severe.



On arranging all in order of severity and dividing into thirds, the group incidence was as follows:

Table I.

	1st third. Slight rickets	2nd third. Medium rickets	3rd third. Severe rickets
I. VD	8	1	1
II. VDX	0	6	4
III. PVD	2	3	4

This experiment, while in keeping with the findings of Hopf and Herman, was open to the objection that the number of animals was small and the dose of VD was not fully curative.

*Exp. 2.* 60 young rats of approximately the same age and from the same mixed stock were used; this experiment was similar to the first except that the dose of the activated and non-activated ergosterol was doubled, namely, 0.0002 mg. per rat per day in 0.1 cc. olive oil. The dose of X-rays was also doubled; the dose of ultra-violet was the same in both experiments, but in this experiment all samples of ergosterol solution were evaporated to dryness before being dissolved in the olive oil; this controlled the possible effect of evaporation on the X-rayed sample in the previous experiment. The X-ray exposure in this case was started  $1\frac{1}{2}$  hours after termination of ultra-violet irradiation. The animals were put on to rachitic diet only 2 days before oil-feeding started. Twelve animals died or were killed before the experiment had been in progress 3 weeks and are not included in the results. The remaining 48 were killed 6 weeks after the experiment had commenced.

The results are expressed below in a similar manner to the first experiment:

Table II.

	Slight	Medium	Severe
I. VD	11	5	2
II. VDX	4	6	3
III. PVD	1	4	12

It is clear that as a result of radiation by X-rays the vitamin D activity of the substance is reduced. Spectroscopic observations have been made and show that, whereas the spectrum of pro-vitamin D is markedly changed after irradiation by ultra-violet light, no change occurs after pro-vitamin D has been submitted to heavy doses of X-rays.

So far as our experiments have gone, they indicate, however, that the spectrum of activated ergosterol is altered when this substance is irradiated with X-rays; the spectrum of the X-irradiated material extending further into the ultra-violet region. Thus it appears that X-rays produce a change in vitamin D which biological experiments show to be of a destructive nature.

The destruction observed in our experiments is only partial but this is not surprising in view of the fact that (1) the degree of activation of the ergosterol used is unknown, (2) the dose of X-rays necessary to destroy the vitamin completely still remains to be established.

## SUMMARY.

1. A dose of 0.0002 mg. of ergosterol activated under the conditions described will partially protect against, and cure, experimental rickets.

2. X-rays when applied to a dry sample of activated ergosterol exposed to the air exert a destructive effect upon vitamin D. This destruction appears to require large doses of X-rays.

## REFERENCES.

- Hopf and Herman (1926). *Sweiz. med. Woch.* 1, 373.  
Rosenheim and Webster (1927). *Biochem. J.* 21, 389.

## CXLII. THE IODINE VALUES OF SOME STEROLS BY DAM'S METHOD.

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*(Received June 30th, 1928.)*

A NUMBER of investigators have pointed out that abnormal iodine values are obtained for sterols and their derivatives when the widely used methods of Wijs or Hübl are employed. Smedley-MacLean and Thomas [1920] (see also Daubney and Smedley-MacLean [1927]) made special reference to the variable values obtained for the unsaponifiable matter of yeast fat.

In the extraction of unsaponifiable fractions, often largely sterol, in this laboratory, a method for iodine values which would give trustworthy results would be of great value. It seemed that the method of Dam [1924] might provide this. Dam's reagent was made up and tested by a series of experiments with cholesterol. Ordinarily 0.25 g. of cholesterol in 10 cc. of chloroform was used, but the method proved trustworthy for 0.025 g. or less, provided that a suitable excess of reagent was used.

Some estimations of the iodine value of pure ergosterol, and also of the sterol content of the unsaponifiable matter of yeast fat were then made. The results were practically the same as those obtained by previous observers.

Ergosterol gave values ranging from 264 to 293 for different samples. On considering these high values there seemed a possibility that they might be accounted for by a fourth double bond, since in this case the theoretical number would be 266, instead of 199 as for three double bonds. Alternatively, the conclusion would be that there is a marked substitution in the ergosterol molecule. In order to throw some light on the matter an investigation of the iodine values of other sterols was undertaken. The results are shown in full in Table 1.

The theoretical iodine value has been calculated for each compound, and, in general, it is found that sterols with one double bond give values approximating most nearly to the calculated result. Also, where there is only one double bond the time of contact with the reagent does not seem to be of great importance.

In the case of cholestenone, which has a ketonic linkage in the molecule, the iodine value is very low, being about half the theoretical value if the solution is treated immediately and even less when the contact with the

reagent is longer. A similar peculiarity is shown by oxycholesterylene which has two double bonds with a ketonic linkage. Stigmasterol with two double bonds gives an approximately correct result, but cholesterylene gives a value of 75 instead of 138. Mauthner and Suida [1896] report similar figures, using Wijs's method. They also state that cholestene with one double bond, and cholesterylene with two give almost identical results.

Table I. *Iodine values of sterols by Dam's method.*

Compound	No. of ethylenic bonds	Amount taken (g.)	Time of contact (mins.)	Iodine value	Theoretical iodine value
Cholesterol	1	0.25	5	65.1	65.5
"	1	0.25	15	65.3	65.5
"	1	0.25	30	67.1	65.5
Cholestenone	1 + C=O	0.25	0	33.0	66.1
"	1 + C=O	0.25	5	31.0	66.1
"	1 + C=O	0.25	15	12.0	66.1
Cholesteryl chloride	1	0.25	0 or 5	62.6	62.7
Cholestene	1	0.25	0	71.9	68.8
Cholesterylene	2	0.0125	0	75.0	138.0
Oxycholesterylene	2 + C=O	0.0125	0	85.0	133.0
"	2 + C=O	0.0125	5	73.0	133.0
Phytosterol	1	0.25	0	66.5	65.5
"	1	0.25	5	70.0	65.5
Sitosterol	1	0.025	0	70.5	65.5
Lupeol	1	0.025	0	68.6	65.5 (?)
"	1	0.025	5	72.3	65.5
Stigmasterol	2	0.0125	2	102.6	119.0
"	2	0.0125	5	126.0	11.9
Ergosterol (a)	3 (or 4)	0.05	0	289.0	199.0 (or 266.0?)
" (a)	3 (or 4)	0.07	5	268.4	199.0
" (a)	3 (or 4)	0.05	15	400.0	199.0
" (b)	3 (or 4)	0.05	0	264.0	199.0
" (b)	3 (or 4)	0.05	5	282.0	199.0
" (c)	3 (or 4)	0.005	0	293.0	199.0
allo-Cholesterol	1	0.025	0	61.8	65.5
allo-Sitosterol	1	0.025	0	71.5	65.5
Coprosterol (a)	0	0.02	0	9.0	—
" (b)	0	0.02	0	7.5	—
$\alpha$ -Cholesterol oxide	0	0.025	0 or 5	2.4	—
$\beta$ -Amyrol	0	0.0107	0	90.2	—
"	0	0.0182	5	90.6	—

When less than 0.05 g. was taken a micro-method was used.

The iodine value of ergosterol was determined on three different samples: (a) as supplied by British Drug Houses, Ltd., (b) as extracted from distillery yeast, and (c) a specially pure yeast ergosterol prepared by Dr Rosenheim. All the values were about 266 or higher, and it was found that the time of contact very definitely affected the values, though with the most rapid titration none lower than 264 could be obtained. A similar result is reported by Smedley-MacLean [1928], who ascribes the high iodine value to a peculiarity of structure rather than to the presence of additional ethenoid linkages.

It seems, however, that structural differences within the molecule do not generally affect the iodine value of a sterol, as is shown by *allo*-cholesterol and *allo*-sitosterol, which give practically the same iodine values as their isomers. This makes it the more difficult to explain the high iodine value of ergosterol. The only other case of extraordinary absorption is that of  $\beta$ -amyrol which,

though a saturated compound, gives an iodine value of 90. The other saturated compounds, coprosterol and  $\alpha$ -cholesterol oxide, give almost negligible iodine values.

Dam [1925] reports also a modification of Wijs's method, in which, by using a suitable excess of the reagent, the time can be reduced to one hour. Using this method in a series of experiments with cholesterol a low value of about 44 was obtained. The result was the same whether the time of contact was one, two, or three hours. Cholestenone and cholesterylene give similarly low values.

#### SUMMARY.

By the pyridine-sulphate-dibromide method of Rosenmund and Kuhnenn [1923] as described by Dam [1925] certain sterols yield abnormal values.

Ergosterol shows a high iodine value which is probably due to substitution and not to the presence of a fourth double bond.

The presence of a ketonic group in the molecule caused a low iodine value in the substances tested.

Structural differences within the molecule of the type represented by *allo*-cholesterol do not cause variations in the iodine values of sterols of the same empirical formula.

Except in the case of ergosterol the duration of the reaction does not greatly affect the iodine value.

In conclusion I desire to thank Prof. Drummond for advice and help, and Dr Rosenheim, Dr Henry, Mr Gardner and Prof. Heilbron for specimens.

#### REFERENCES.

- Dam (1924). *Biochem. Z.* **152**, 101.  
— (1925). *Biochem. Z.* **153**, 76.  
Daubney and Smedley-Maclean (1927). *Biochem. J.* **21**, 869.  
Mauthner and Suida (1896). *Monatsh.* **17**, 29.  
Rosenmund and Kuhnenn (1923). *Z. Untersuch. Nahr. Genussm.* **46**, 154.  
Smedley-MacLean (1928). *Biochem. J.* **22**, 23.  
Smedley-MacLean and Thomas (1920). *Biochem. J.* **14**, 483.

# CXLIII. THE SELECTIVE FERMENTATION OF GLUCOSE AND FRUCTOSE BY BREWER'S YEAST.

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*(Received July 4th, 1928.)*

THE literature dealing with the subject of the selective fermentation of mixtures of glucose and fructose by living yeast contains many apparently contradictory and confusing statements. A summary of the earlier work is given by Lippmann [1904]. The description "fermentation élective" was used by Dubrunfaut and this title has frequently been used by subsequent workers. It is well established by the work of Slator [1908] that living yeast of various species and in various conditions of activity ferments glucose and fructose separately at approximately the same rate. The combined rates of fermentation of the two sugars when mixed is also this same rate, but the relative rates of disappearance of the respective sugars from a mixture of the two undergoing fermentation varies very widely with the species of yeast used and with other conditions. Thus, Sauterne yeasts are stated to ferment fructose faster than glucose, whereas with many yeasts, including *S. cerevisiae*, the reverse phenomenon has been observed. Most of the experimental evidence upon which these statements are founded has been the specific rotation of the mixed sugars remaining in the partly fermented solution. However, Schiller [1925] used the iodimetric method of Willstätter and Schudel as modified by Judd [1920] and by Baker and Hulton [1920] and obtained evidence of selectivity in various yeasts.

It was considered that it was of primary importance to confirm these phenomena of selective fermentation by methods other than those based upon specific rotation, and to consider any alternative explanations. It was shown by Neuberg and Kobel [1926] that when fructose is mixed with certain amino-acids an immediate change in rotation occurs, in the negative direction. Whilst such changes as were recorded are of an order quite inadequate to provide a simple explanation of the specific rotations of partly fermented solutions of invert sugar, they justify a careful reconsideration of the latter results. The possibility also arises that the nitrogen compounds excreted by the yeast during fermentation may possess an independent and appreciable specific rotation. Even when the yeast is vigorously reproducing, some excretion occurs, if only from old and dead cells which are inevitably present.

Unfortunately these substances absorb iodine and thus interfere with the iodimetric determinations of glucose, and their complete removal by precipitating reagents is not easy.

Solutions of (a) glucose, (b) fructose and (c) an equal mixture of the two sugars were fermented under exactly similar conditions with brewery (top) yeast (Exp. 1) and it was found that, throughout the fermentation:

(1) the specific rotation of the unfermented sugar in

(a) remained approximately  $+52^{\circ}$ ;

(b) remained approximately  $-92^{\circ}$ ;

(c) fell steadily from  $-19.6^{\circ}$  to  $-50^{\circ}$ :

(2) in each of the three solutions the percentage of glucose obtained by iodimetric titration was in fair agreement with that obtained from rotation and reducing power, but exceeded it by a similar small amount in all cases. The fact that the glucose results obtained iodimetrically exceeded those obtained polarimetrically was due to the presence of excreted nitrogen compounds which could not be completely removed by such agents as normal lead acetate, phosphotungstic acid and animal charcoal. Since the three fermentations were performed under similar conditions, it may be presumed that the yeast excretions were equivalent in all three at the corresponding stages of the fermentations.

The above results were obtained at four stages of each fermentation. Multiplication of yeast occurred in these fermentations since malt culm extract was added as nutrient, but exactly similar results were obtained in three other fermentations, in one of which yeast water, in another synthetic nutrients, were employed and in the other no nutrients were added. In the last case more yeast autolysis and excretions might be expected, but the general tenor of the results was not affected. For evident reasons the iodimetric method could not be employed as a check where synthetic nutrients, which included ammonium salts, were used.

From these experiments it seems fairly evident that, under the respective conditions adopted, this brewery yeast removes glucose more rapidly than fructose from a solution containing equal quantities of the two. Similar results were also obtained using Burton brewery yeast (more attenuative) and Lager (bottom) yeast.

In some of the investigations the percentage of fructose present was confirmed by measuring the rotation at  $20^{\circ}$  and  $82^{\circ}$  respectively, and the results obtained were in fair agreement with the others.

In explanation of the phenomena so far described one needs to reconcile three apparently contradictory facts:

(1) that glucose and fructose in separate solutions are fermented at approximately equal rates by living yeast:

(2) that glucose and fructose in mixtures are fermented at unequal rates, glucose being fermented the faster by most yeasts;

(3) that fructose alone is specifically esterified at least as fast as glucose by yeast juice or zymine, and more rapidly than glucose in the presence of suitable concentrations of added phosphates [Harden and Young, 1909].

The work of Slator [1908] and Slator and Sand [1910] indicates that the rate of fermentation of sugars by living yeast depends upon factors operating within the cell, the rate of diffusion inwards being, except in very dilute solutions, such as to supply sugar faster than it can be dealt with inside. In view of this and of the fact that fermentation within living cells occurs at rates higher than those obtainable with corresponding quantities of non-living yeast preparations, even when phosphates are added, one would expect fructose to be fermented faster than glucose. This is not the case, and, as far as separate fermentations are concerned, the simplest explanation is that one reaction ultimately controls the rate of fermentation in all these cases. This reaction is, according to the theory of Harden and Young [1908], a splitting of hexosediphosphate by the enzyme hexosephosphatase, thereby releasing free phosphate to be used in esterifying further sugar. The constancy of the rate of fermentation over an appreciable period of time points to this being a typical enzyme action, the concentration of both the enzyme (hexosephosphatase) and of its substrate (hexosediphosphate) remaining approximately constant. It is the existence of this cycle of changes which maintains the uniform velocity of the reaction. The hexosediphosphate concentration is limited by the phosphorus content of the cell. In such circumstances a relatively rapid esterification of fructose as compared with that of glucose will be of no appreciable significance when the rates of fermentation of the separate hexoses are under consideration.

An explanation of the selective fermentation of glucose and fructose involves many considerations. The relative rates of diffusion of these two sugars through the cell membrane have been held by some writers to account for the phenomenon, but diverse opinions have been expressed. Slator [1908] summed up by saying, "It is improbable that the somewhat different rates of diffusion of dextrose and laevulose into the cell play any important part in the determining whether dextrose or laevulose disappears faster from the solution." In addition to the evidence available at that time we have the observation that the zymine prepared from brewery yeast exhibits the same phenomenon as the yeast itself in this respect [Fernbach, Schoen and Mori, 1927]. (See also Exp. 4.) Lastly, if the specific rates of diffusion were the only consideration we should have to postulate that glucose diffuses two and a half times as fast as fructose through the cell membrane of brewery yeast in certain cases, whilst fructose diffuses from two to three times as fast as glucose through the cell membrane of (*e.g.*) Sauterne yeasts. It is very much more unlikely that cell membranes should so differ, than that, for example, the relative activities of the various enzymes within the cells should differ.

It was suggested by Slator [1911] that when glucose alone is fermented by living yeast, fructose may be excreted from the cell and found in the partly



fermented solution. Attempts to obtain evidence of this under various conditions were unsuccessful (Exps. 1 to 3). To test for fructose, the specific rotation, the change of rotation of fructose with change of temperature, the colour reaction of fructose with orcinol and phosphoric acid [Löwe, 1916] were used. From this it appears that the excess of fructose found in partly fermented solutions of invert sugar does not owe its presence to excretions of fructose from within the cell.

Further experiments showed that the selective fermentation by top and bottom brewery yeasts is approximately independent of:

- (a) concentration of total sugars between the limits 3.6 % and 19 %;
- (b) temperature between the limits 12° and 26°;
- (c) reaction of medium between the limits of  $p_H$  4.0 and 7.5, whether nutrient and buffering substances are present or not;
- (d) the fermentative power of the yeast as modified by the action of heat.

The relative rates of disappearance of the two sugars change as fermentation proceeds, since the proportion of the sugars in the medium is constantly changing. The ratio of the two specific rates is however more constant, and was calculated by means of the formula

$$K = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)},$$

where

$a_g$  = initial concentration of glucose,

$a_f$  = initial concentration of fructose,

$(a_g - x_g)$  = concentration of glucose at time  $t$ ,

$(a_f - x_f)$  = concentration of fructose at time  $t$ .

This formula was obtained by the following considerations. The formula for the velocity constant of a monomolecular reaction  $k = \frac{1}{t} \ln \frac{a}{a-x}$  has often been misapplied to fermentations of single sugars by living yeast, but the formula is applicable relatively to the individual sugars in selective fermentations. Whilst the combined rate of fermentation of the two sugars is uniform for a considerable period if yeast concentration remains constant, for each individual sugar the rate of fermentation at any instant will be proportional to its concentration at that instant and the law will hold. If  $k_g$ ,  $a_g$  and  $x_g$  represent the values of  $k$ ,  $a$  and  $x$  respectively for glucose at time  $t$ , and  $k_f$ ,  $a_f$ ,  $x_f$  those of fructose, then

$$\frac{k_g}{k_f} = \frac{\frac{1}{t} \ln \frac{a_g}{a_g - x_g}}{\frac{1}{t} \ln \frac{a_f}{a_f - x_f}} = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)} = K,$$

which might be regarded as the "selectivity constant." This value, however, appears to increase somewhat during fermentation except in the very late stages when it approaches unity.

Using solutions containing approximately equal proportions of glucose and fructose, and a top brewery yeast, the following values of  $K$  were obtained:

18.0 % sugar (initially)	1.78	} at 25°;
9.5 %        ,,	1.81	
3.6 %        ,,	1.79	
3.6 %        ,,	1.88	at 12°;
3.6 %        ,,	1.76	at 25° and at $p_H$ 7.0 throughout;
3.6 %        ,,	1.68	at 25° using yeast, 60 % of the fermentative power of which had been destroyed by heat;
3.6 %        ,,	1.80	at 25° using yeast which had been grown in a medium of fructose and nutrients.

The value of this constant does not appear to vary much beyond the limits of experimental error under the conditions. Perhaps selectivity is rather greater at low temperatures, a result obtained by Bourquelot [1885], and less in yeast of which the activity has been reduced by heat. Far greater differences exist between yeasts of different species, and yeasts under different conditions of nutrition. Whilst the Edinburgh brewery top yeast above gives the value  $K = 1.8$  under normal conditions, a bottom yeast gave  $K = 2.1$  at 20° under similar conditions, and the top yeast, when growing in synthetic nutrients rich in phosphates (see Exp. 3), gave  $K = 2.8$  at 20°. From the results obtained by Schiller [1925] calculation shows  $K$  to be 0.5 or less for a Sauterne yeast, in which case the fructose disappears from the medium faster than the glucose.

The reaction of the medium was found by Schiller to have a definite influence, the fructose being fermented relatively faster in more alkaline media. This was not found to be the case with the two types of brewery yeast referred to above. Schiller's work seems to be open to criticism however. He fermented mixtures of glucose and fructose with various yeasts, using malt culm extract as nutrient and measuring glucose at intervals by the iodimetric method. The fructose was estimated by deducting the quantity of glucose so obtained from the total reducing sugar as measured by Bertrand's method. The nitrogenous compounds present in the malt culm extract were removed by treatment with animal charcoal. The results obtained indicated that, with most yeasts, whilst glucose is the more rapidly fermented in the more acid media, selectivity is less pronounced in more alkaline media, and, indeed, in the early stages of fermentation in alkaline media the fructose is the more rapidly removed of the two. On repeating Schiller's work with top and bottom brewery yeasts it was found that animal charcoal does not remove nitrogenous material excreted by the yeast, and that somewhat high results for glucose are thereby obtained by the iodimetric method. Further it was found that, on boiling solutions of  $p_H$  7.0, if fructose is present this sugar is altered to some extent, the resulting products possessing a less negative rotation

and a higher iodine value than before boiling. During boiling of the partly fermented solutions, a procedure necessary to remove volatile matters before estimating the reducing sugars, carbonic acid is evolved and the liquid becomes more alkaline. If the reaction is beyond  $p_H$  7.0 during boiling, the subsequent analysis will yield high results for glucose both polarimetrically and iodimetrically. These observations may be the explanation of Schiller's results, which could not be obtained with brewery yeast.

The possibility that yeasts which ferment fructose faster than glucose may have acquired such characteristics from long habitat in media rich in fructose suggested that a brewery yeast, normally living in wort rich in maltose, might on repeated cultivation in a medium containing fructose be "trained" to ferment fructose selectively faster than glucose. Such subculturing was continued for several weeks, but the results indicated no change in the properties of the yeast in this respect.

Experiment showed that, for the zymins prepared from the top and bottom brewery yeasts previously referred to, the ratio of *maximum* rates of esterification attained (as measured by  $CO_2$  evolution) for fructose : glucose,

(1) increased with concentration of free phosphate when this was above a certain concentration for each zymine under the conditions adopted;

(2) did not vary between  $15^\circ$  and  $30^\circ$  when the phosphate concentration was that of the optimum for glucose;

(3) did not appreciably vary between  $p_H$  5.9 and 6.8 when the phosphate concentration was that of the optimum for glucose.

Of these three the influence of phosphate concentration is the most important. With the zymine from the top yeast for example the ratio fructose : glucose was 1.1, for concentration of free phosphates within 0.15 *M*. Above this the ratio increased to 1.4 with 0.19 *M* phosphate. Comparatively slight variations in phosphate concentration over this zone have an appreciable effect on the ratio. With yeast juice, which shows greater susceptibility to phosphates, wider variations in the ratio are to be expected. This is illustrated by the results obtained by Harden and Young [1909]. One possible inference from these results, assuming the hexosephosphate theory of Harden and Young, is that variations in species and in conditions of nutrition of yeast, reflected by variations in the relative activity of hexosephosphatase and of the constituents of the esterifying complex, would be a factor in accounting for the different values of *K* in selective fermentations of glucose and fructose. Temperature and reaction of medium would have comparatively little influence.

The temperature coefficient of esterification by the zymine was found to be  $\frac{V_{25^\circ}}{V_{15^\circ}} = 2.01$  for glucose with the optimum phosphate addition, and that for fructose 2.08 with the same phosphate addition. It is by no means certain that this coefficient would hold for different phosphate additions. These results are somewhat higher than those obtained by Euler and Ohlsén [1911] using sucrose and dried yeast extract. These authors measured the rate of dis-

appearance of free phosphate at 17.5° and 30° respectively. This however does not give the true temperature coefficient of esterification, since the continuous re-formation of free phosphate by the hexosephosphatase causes the rate of disappearance of free phosphate measured to be less than the true one. The temperature coefficient for hexosephosphatase  $\frac{V_{24.5^\circ}}{V_{14.5^\circ}}$  was found to be 2.88 by Herzog (which was roughly confirmed in conjunction with the above experiments with zymine). It is in good agreement with that for yeast cells as found by Slator [1908]  $\frac{V_{25^\circ}}{V_{15^\circ}} = 2.80$ . Since this coefficient exceeds that of esterification, the velocities observed by Euler and Ohlsén would be below the actual at each temperature, and in greater proportion at the higher temperature than at the lower one, and consequently the temperature coefficient obtained by them must be lower than is actually the case.

The approximate equality of the temperature coefficient of esterification of glucose and fructose in the presence of small concentrations of free phosphate is not surprising since the same enzyme functions for both sugars. But the abnormal properties of fructose, and in particular its unique change in specific rotation with rise of temperature, suggest that an internal structural change is occurring in one of the isomeric ( $\alpha$ ) forms and that this change may be such as to produce a more or less labile form of the sugar, with possible effects upon the velocity of esterification.

The influence of the reaction of the medium between the limits of  $p_H$  3.5 and 7.5 upon the  $p_H$  of the cell interior is very uncertain, but it is probably comparatively little. One would not then expect any decisive influence to be exerted upon selective fermentation in view of the esterification ratios found over the range of  $p_H$  5.9 to 6.8.

#### EXPERIMENTAL.

*Exp. 1. The sugars remaining in solution during the fermentations of glucose, fructose, and a mixture of the two by brewery (top) yeast.*

Solutions of these sugars, boiled to destroy mutarotation, were treated with 5 % of fermented and boiled extract of malt culms to serve as yeast nutrient, and seeded with equal seed rates of pure culture yeast. Half of each was rendered faintly alkaline,  $p_H$  7.5, and maintained at  $p_H$  7.0 to 7.5 throughout the fermentation by occasional small additions of concentrated alkali. The fermentations were conducted at 20°. Samples were withdrawn at the intervals stated, filtered, if alkaline rendered faintly acid, boiled to destroy any possible mutarotation and to remove volatile reducing substances and made up to the original volume. Rotations were measured at 20° in a 200 mm. tube and reducing powers by the method of Lane and Eynon (using methylene blue). The iodimetric titrations were performed exactly as described by Baker and Hulton [1920]. The use of alumina cream, which is liable to contain ammonium salts, must be avoided in the solutions used for the

iodimetric titration. Allowance was made for the small absorption of iodine by fructose.

The solutions containing the residual sugars after 70 hours' fermentation were also read in the polarimeter at 82°, at which temperature an equal mixture of the two sugars should have no rotation, whilst the specific rotation of fructose should have fallen to about -52° and that of glucose should remain unchanged. The percentage fructose was calculated from the difference between the readings at 20° and 82° on the assumption that the specific rotation of fructose decreases by 0.6385° per degree rise in temperature. It was found necessary to maintain the polarimeter tube in its bath at 82° for over 1 hour to obtain constant readings. On cooling the rotations returned to normal.

Table I.

Sugar fermented	Glucose		Fructose		Glucose and fructose	
	5.0-4.0	7.5-7.0	5.0-4.0	7.5-7.0	5.0-4.0	7.5-7.0
$p_H$ during fermentation						
Original solution. Conc. of sugar (g. per 100 cc.)	7.9	7.9	7.85	7.85	Glucose 4.20 Fructose 4.20	4.20 4.20
$[\alpha]_D^{20}$ after 16 hours	+51.7°	+51.6°	-92.5°	-92.5°	-23.8°	-23.9°
Conc. of sugar (g. per 100 cc.)	7.55	7.38	6.50	6.35	Glucose 3.35 Fructose 3.75	3.31 3.72
Iodine absorption expressed as glucose (g. per 100 cc.)	7.65	7.50	0.07	0.12	3.48	3.45
$[\alpha]_D^{20}$ after 22 hours	+51.5°	+51.5°	-92.1°	-91.7°	-27.0°	-25.8°
Conc. of sugar (g. per 100 cc.)	6.95	5.90	5.58	5.44	Glucose 2.74 Fructose 3.36	2.76 3.26
Iodine absorption expressed as glucose (g. per 100 cc.)	7.10	6.10	0.14	0.18	2.91	2.98
$[\alpha]_D^{20}$ after 46 hours	+50.6°	+49.9°	-94.0°	-92.6°	-34.5°	-35.7°
Conc. of sugar (g. per 100 cc.)	6.25	4.21	3.90	3.85	Glucose 1.71 Fructose 2.61	1.62 2.55
Iodine absorption expressed as glucose (g. per 100 cc.)	6.45	4.44	0.18	0.28	1.92	1.85
$[\alpha]_D^{20}$ after 70 hours	+50.1°	+49.7°	-93.0°	-94.5°	-49.4°	-49.4°
Conc. of sugar (g. per 100 cc.)	4.80	3.15	2.35	3.02	Glucose 0.70 Fructose 1.68	0.70 1.68
Iodine absorption expressed as glucose (g. per 100 cc.)	5.02	3.40	0.26	0.24	0.94	0.96
$[\alpha]_D^{82}$	+50.0°	+49.6°	-55.0°	-56.2°	-22.4°	-22.2°
Fructose calculated from $[\alpha]_D^{20}$ and $[\alpha]_D^{82}$ (g. per 100 cc.)	0.0	0.0	2.25	2.92	1.62	1.63

*Exp. 2.* A series of fermentations as in *Exp. 1* was performed without added nutrients with results similar to those in *Exp. 1*.

*Exp. 3* (Table II). A mixture of glucose and fructose was fermented at 20° in the presence of the following synthetic nutrients:

$\text{NH}_4\text{NO}_3$	3.0 g. per litre	$\text{Na}_2\text{HPO}_4$	6.9 g. per litre
$\text{MgSO}_4$	0.75 "	$\text{ZnSO}_4$	Trace
$\text{KH}_2\text{PO}_4$	0.6 "	$\text{FeSO}_4$	"

The phosphate content of this medium was  $M/20$ . Included in the table are the values of  $K$ , the ratio of the specific rates of fermentation (glucose to fructose).

Table II. *Concentration of sugars present in g. per 100 cc.*

	Original solution	Hours				
		22	31	46	73	145
Glucose	7.50	6.41	5.62	4.45	2.90	0.20
Fructose	7.50	7.09	6.78	6.25	5.27	0.90
$K$	—	2.78	2.86	2.87	2.70	1.71

A similar fermentation of glucose alone with the same yeast and nutrients gave successive specific rotations:  $+53.0^\circ$ ,  $+51.1^\circ$ ,  $+51.1^\circ$ ,  $+49.6^\circ$ ,  $+50.0^\circ$ . The solutions were tested for fructose by means of the reaction with orcinol and phosphoric acid [Löwe, 1916], but no evidence of the presence of fructose was obtained, whereas control tests with 0.1% of fructose added gave positive results.

*Exp. 4. Fermentation of mixtures of glucose and fructose by zymin.*

75 cc. of solutions containing 5 g. of glucose and 5 g. of fructose were fermented with 10 g. of zymin prepared from

- (a) top fermentation brewery yeast,
- (b) bottom fermentation brewery yeast,

with toluene, without added phosphates, at  $25^\circ$  for 24 hours. The liquids were now partly cleared with alumina cream, diluted and boiled until all volatile substances were evolved, and cleared afresh with alumina cream. The rotations and reducing powers were determined, and calculating the latter as percentage of glucose the following specific rotations were obtained:

Using top yeast zymin  $[\alpha]_D^{20} - 28.0^\circ$ .

Using bottom yeast zymin  $[\alpha]_D^{20} - 25.5^\circ$ .

These rotations would have been more negative but for the presence of a substance of high dextrorotatory power and low reducing power such as was shown to be present by Harden and Young [1913]. The solutions were next fermented with living yeast as completely as possible and the above process repeated. The specific rotations were now  $+112^\circ$  in each case. Some fermentable sugars and hexosephosphates would still be present, as it is difficult to ferment away the last traces of sugar, and hexosephosphates are apparently not fermentable by living yeast. It is fairly evident that the fermentable sugars remaining after the partial fermentation by zymin had a specific rotation more negative than the figures  $-25.5^\circ$  and  $-28^\circ$ , which were obtained in the presence of the highly dextrorotatory polysaccharide.

*Velocities of esterification of glucose and fructose by zymin.*

The velocity of esterification was measured by determining the loss of  $\text{CO}_2$ . The zymin was added to the sugar solution in U-tubes (all of similar shape

and size) with well-fitting taps, and the side tubes plugged lightly with cotton wool. After incubation until the fermentation was steady and the system saturated with  $\text{CO}_2$ , the phosphate (saturated with  $\text{CO}_2$ ) was added and the air in the tube displaced by passing through a current of  $\text{CO}_2$ . The taps were closed when weighing and when shaking. Weighings were taken as frequently and rapidly as possible, every few minutes, and losses of weight plotted graphically against time. After a short induction period, longer with glucose than with fructose, the velocity of gas evolution soon acquired a maximum which remained uniform for some time, depending upon the quantity of phosphate added. The curve here was remarkably straight, and the maximum rates were read off from this part of the curve. The losses of weight were all corrected for water vapour.

*Exp. 5. Velocity of esterification of glucose and fructose by zymine in presence of varying concentrations of phosphates.*

To 2 g. of zymine were added 8 cc. of a solution containing 2 g. of the sugar and 0.2 cc. toluene and the mixture was incubated at  $25^\circ$  until a constant rate of fermentation had been attained. The quantities of 0.6  $M$   $\text{K}_2\text{HPO}_4$  (saturated with  $\text{CO}_2$ ) were added and water (saturated) to total volume 15 cc. The rates of gas evolution are recorded in mg. per 5 mins. (1 mg.  $\text{CO}_2$  = approx. 0.5 cc.).

Table III.

cc. 0.6 <i>M</i> K <sub>2</sub> HPO <sub>4</sub>	Max. rate, mg. per 5 mins.		Ratio F./G.	Average rate over whole acceleration period		Ratio F./G.
	Glucose	Fructose		Glucose	Fructose	
0	Too transient for accurate measurement					
2.0	6.76	7.57	1.12	4.8	5.30	1.10
3.0	7.50	8.10	1.08	5.7	6.16	1.08
3.5	8.30	9.00	1.08	5.8	6.25	1.08
4.0	8.25	9.36	1.13	5.8	6.30	1.09
5.0	7.37	10.40	1.41	5.7	7.06	1.24
6.0	7.16	10.65	1.50	5.5	7.85	1.43

*Exp. 6. Temperature coefficient of esterification of glucose and fructose.*

2 g. zymine + 10 cc. of solution containing 2 g. sugar were incubated as before with toluene. 3.5 cc. 0.6  $M$  phosphate and water to 15 cc. were added.

Table IV.

Temperature	Max. rates attained, mg. per 5 mins.		
	$15^\circ$	$25^\circ$	$30^\circ$
Glucose	2.55	5.12	7.15
Fructose	4.83	10.05	13.90
	$V_{25^\circ}$	$V_{30^\circ}$	$V_{30^\circ}$
	$V_{15^\circ}$	$V_{25^\circ}$	$V_{15^\circ}$
Glucose	2.01	1.40	2.80
Fructose	2.08	1.37	2.88

*Exp. 7. Influence of hydrogen ion concentration on the relative velocities of esterification of glucose and fructose.*

The hydrogen ion concentrations in this experiment were measured electrometrically, by the quinhydrone electrode, using the contents of control tubes

which were in all cases started at a known interval of time after the ordinary weighed tubes. A sample was withdrawn from the control tube just as the maximum rate of esterification was being attained, the time of this being the known interval after the contents of the weighed tube had arrived at the same stage. Another sample was withdrawn at the end of the period of esterification. The hydrogen ion concentration was determined immediately and rapidly in each case. The maximum velocities recorded do not represent the true maxima for the respective hydrogen ion concentrations, since a certain amount of  $\text{CO}_2$  is released from the system as a result of the change in degree of ionisation of the phosphates during esterification. This amount is assumed to be identical for glucose and fructose in any one case (*i.e.* over the same range of  $p_{\text{H}}$ ), since the same amount of free phosphate is esterified and the change in  $p_{\text{H}}$  should be the same. The *ratios* should not be affected. Further work is required to ascertain the true rates of esterification at the respective ranges of  $p_{\text{H}}$ . The concentration of phosphate added was the optimum for glucose under normal conditions.

To 2 g. zymin were added 10 cc. of solution containing 2 g. of sugar, and after incubation 3.5 cc. of 0.6 *M* phosphate, etc. (as stated in Table V), and the total volume was made up to 15 cc.

Table V.

cc. of 0.6 <i>M</i>				Max. rate, mg. $\text{CO}_2$ per 5 mins.			$p_{\text{H}}$
$\text{K}_2\text{HPO}_4$	$\text{KH}_2\text{PO}_4$	$\text{KHCO}_3$	$\text{KCl}$	Glucose	Fructose	Ratio F./G.	
1.2	2.3	0	1.5	5.00	5.40	1.08	6.04-5.91
1.5	2.0	0	1.5	5.40	6.06	1.12	6.21-6.06
2.0	1.5	0	1.5	6.80	7.80	1.15	6.30-6.20
2.8	0.7	0	1.5	7.95	9.15	1.15	6.36-6.30
3.5	0	0	1.5	8.90	10.70	1.20	6.60-6.50
3.5	0	1.0	0.5	10.50	13.30	1.26	6.88-6.80

## SUMMARY.

1. Brewery yeasts (top and bottom types) ferment glucose faster than fructose in mixtures of the two, both when the yeast is multiplying freely in the presence of various kinds of nutrients, and when nutrients are absent.

2. The relative specific rates of fermentation of glucose and fructose in such cases are far more dependent upon the species of yeast used and the conditions of its nutrition, than upon such factors as concentration of sugars in the medium, temperature, and reaction of medium.

3. No fructose could be detected in a solution of glucose undergoing fermentation by living yeast.

4. Zymin, prepared from brewery yeast, ferments glucose faster than fructose in a mixture of the two.

5. A yeast "trained" by continual subculturing in a medium of fructose and nutrients showed no change in its properties as regards selectivity.



6. The temperature coefficients of esterification of glucose and fructose by zymase under conditions of phosphate concentration yielding the optimum esterification of glucose are approximately the same, viz.  $\frac{V_{25^\circ}}{V_{15^\circ}} = 2.0$ .

7. The velocities of esterification of both sugars vary with the reaction of the medium, but the ratio of the velocities varies much less.

In conclusion I wish to thank Prof. A. Harden for useful criticisms of this work during the investigation.

#### REFERENCES.

- Baker and Hulton (1920). *Biochem. J.* **14**, 754.  
 Bourquelot (1885). *Compt. Rend. Soc. Biol.* 223.  
 Euler and Ohlson (1911). *Biochem. Z.* **37**, 318.  
 Fernbach, Schoen and Mori (1927). *Ann. Brass. Dist.* **25**, 226.  
 Harden and Young (1908). *Proc. Roy. Soc. Lond.* **80**, 299.  
 ——— (1909). *Proc. Roy. Soc. Lond.* **81**, 336.  
 ——— (1913). *Biochem. J.* **7**, 630.  
 Judd (1920). *Biochem. J.* **14**, 255.  
 Lippmann (1904). *Chemie der Zuckerarten*, 1, 934.  
 Löwe (1916). *Proc. Soc. Exp. Biol. Med.* **13**, 71.  
 Neuberg and Kobel (1926). *Biochem. Z.* **174**, 464.  
 Schiller (1925). *Ann. Brass. Dist.* **23**, 197, 215, 228.  
 Slaton (1908). *J. Chem. Soc.* **93**, 238.  
 ——— (1911). *J. Inst. Brew.* **17**, 147.  
 Slaton and Sand (1910). *J. Chem. Soc.* **97**, 922

# CXLIV. THE METABOLISM OF TRYPTOPHAN.

## II. SYNTHESIS OF 6-METHYL- AND 8-METHYL-KYNURENIC ACIDS.

By WILLIAM ROBSON.

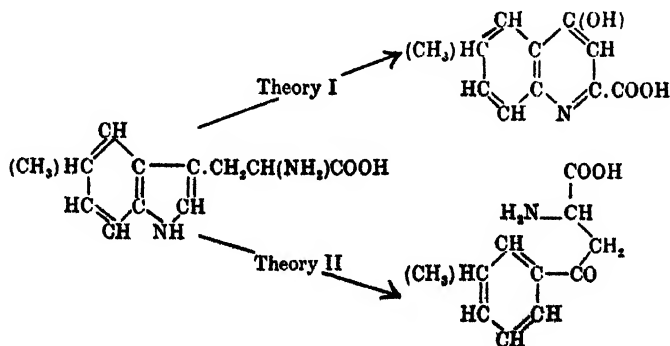
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(Received July 3rd, 1928.)

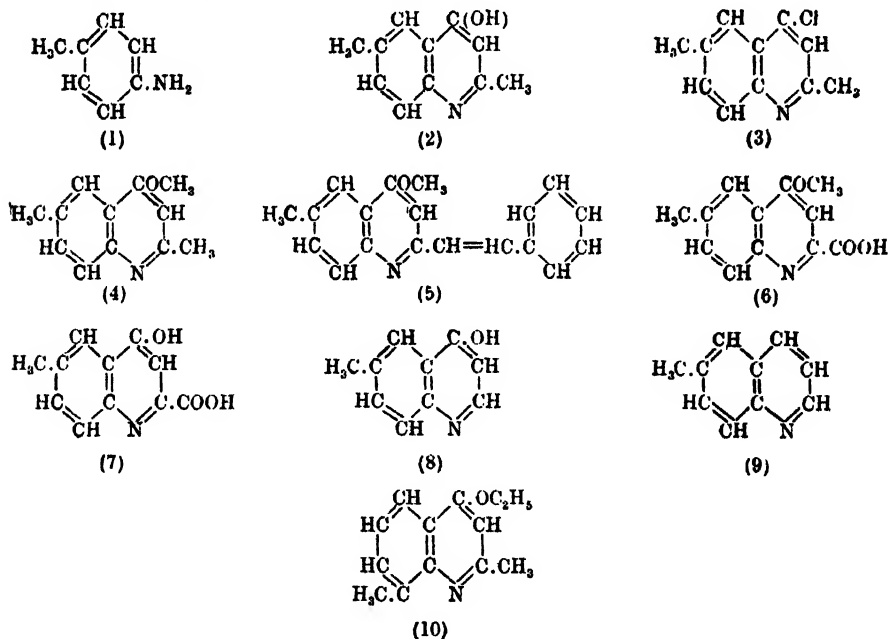
THE mechanism of the mode of formation of kynurenic acid from tryptophan in the animal body is not yet fully understood. Of the various ways of attempting the elucidation of this problem it has been suggested that a substituted tryptophan would possibly yield a substituted kynurenic acid, and feeding experiments on these lines were carried out with *Pr*-2-methyltryptophan by Ellinger and Matsuoka [1914] and Barger and Ewins [1917]. In neither case was a substituted kynurenic acid found in the urine of the animal. From the results of their experiments, however, the latter workers concluded that a tryptophan substituted in the benzene nucleus would probably give the desired result. This idea formed the basis of the present investigation.

For reasons advanced in a former paper the author [1924] showed that the most suitable of the many such substituted tryptophans theoretically possible for such a study was *Bz*-3-methyltryptophan. In the same communication the synthesis of this compound was described.

The first problem which presents itself regarding the formation of kynurenic acid is whether its single nitrogen atom is that of the pyrrole nucleus (Theory I), or that of the amino-group (Theory II) of the tryptophan molecule.



If either of these theories is correct, it follows that the feeding of *Bz-3*-methyltryptophan to a dog or to a rabbit should result in the appearance in the urine of the animal of 6-methylkynurenic acid (Theory I) or 8-methylkynurenic acid (Theory II). The isolation, therefore, of either of these acids and its identification by degradation to the corresponding known methylquinoline would, at first sight, appear to accomplish the aim of the investigation. There is, however, a serious drawback to such a mode of procedure. Of the behaviour in the animal organism of compounds of quinoline having structures resembling those of the above methylkynurenic acids little is known. Reference to the literature shows that the only work bearing any relation to this aspect of the investigation was done by Cohn [1895] who studied the fate of certain methylquinolines in the dog and in the rabbit. Of the three methylquinolines which he injected he found that, whereas quinaldine (2-methylquinoline) and *o*-methylquinoline (8-methylquinoline) appeared to be completely destroyed in the animal organism. *p*-methylquinoline (6-methylquinoline) was excreted in the form of 6-carboxyquinoline. The disparity in the behaviour of these compounds made it appear essential that the present investigation should include a study of the fate of the two methylkynurenic acids, which, as shown above, are possible end-products of the metabolism of *Bz-3*-methyltryptophan. For that purpose the synthesis of both these acids was undertaken and is described below.



The schemes of synthesis of 6-methylkynurenic acid and 8-methylkynurenic acid had as their starting-points *p*-toluidine and *o*-toluidine respectively, and

resembled in certain respects that followed by Besthorn [1921] in his synthesis of kynurenic acid from methoxyquinaldine. *p*-Toluidine (1) was condensed with ethyl acetoacetate, the product of this reaction on heating yielded *p*-methyl- $\gamma$ -hydroxyquinaldine (2). The latter compound readily gave a corresponding chloro-compound (3), which reacted with sodium methoxide to form *p*-methyl- $\gamma$ -methoxyquinaldine (4).

Condensation of this compound with benzaldehyde resulted in the elimination of water and the formation of benzylidene-*p*-methyl- $\gamma$ -methoxyquinaldine (5). In his synthesis, Besthorn, for the purpose of introducing a carboxy-group in the  $\beta$ -position (pyridine ring), condensed methoxyquinaldine with formaldehyde, and oxidised the resulting formyl compound with nitric acid. In the present instances it was thought that, under the directive influence of the methyl group, there was a distinct possibility of the use of nitric acid leading to the nitration of the benzene ring. To avoid this, use was accordingly made of potassium permanganate in acetone solution, when a good yield of *p*-methyl- $\gamma$ -methoxyquinoline-2-carboxylic acid (6) was obtained. Replacement of the methoxy-group in this compound by a hydroxy-group—an operation accomplished by heating with constant-boiling hydrobromic acid—led to the formation of *p*-methyl- $\gamma$ -hydroxyquinoline-2-carboxylic acid or 6-methylkynurenic acid (7). The constitution of the acid so obtained was established in the following manner. Heating the 6-methylkynurenic acid at its melting-point led to loss of carbon dioxide and the formation of 6-methyl- $\gamma$ -hydroxyquinoline (8), which compound, on heating with zinc dust, gave 6-methylquinoline (9) which is known in the literature.

The synthesis of 8-methylkynurenic acid followed similar lines.

#### EXPERIMENTAL.

*p*-Methyl- $\gamma$ -hydroxyquinaldine (formula 2). This compound, as well as *o*-methyl- $\gamma$ -hydroxyquinaldine, was synthesised by Conrad and Limpach [1888] and their method was closely followed. Equimolecular quantities of commercial *p*-toluidine (107 g.) and ethyl acetoacetate (130 g.) were placed in a stoppered reagent bottle and allowed to stand at room temperature for 5 days. An aqueous layer separated and this was removed by distilling the mixture under reduced pressure on the water-bath. The clear oil remaining was transferred to a second distillation flask and rapidly heated to 230°, when the flame was lowered. A violent reaction set in and the temperature rose to 250°. The heating was continued for 10 minutes after the reaction had subsided and the residual highly coloured fluid was quickly poured into a large evaporating basin where it gradually solidified into a dark brown mass. This, when cold, was broken up, boiled with dilute hydrochloric acid and much charcoal and filtered through a hot-water funnel. On cooling, the slightly coloured liquid was neutralised with solid sodium carbonate when a straw coloured crystalline mass separated. This, after standing for 24 hours, was filtered off, dissolved in a large quantity of boiling water to which charcoal had been added, and the

hot solution again filtered. The solution when cold deposited 20 g. of *p*-methyl- $\gamma$ -hydroxyquinaldine. This was washed with water and dried in the air-oven at 130°. The dry compound melted at 279–280°. (Conrad and Limpach quote *p*-methyl- $\gamma$ -hydroxyquinaldine as melting at 274–5°.)

*o*-Methyl- $\gamma$ -hydroxyquinaldine. The preparation of this compound from *o*-toluidine and ethyl acetoacetate followed exactly the same lines as those described above for the synthesis of its *para*-isomer. The yield, however, was higher, 30 g. being obtained from the same quantities of starting materials. The melting-point of the product was 260–1°, which is that given by Conrad and Limpach.

*p*-Methyl- $\gamma$ -chloroquinaldine (formula 3). Following the method of Lieben and Haitinger [1885] who prepared  $\gamma$ -chloropyridine from  $\gamma$ -hydroxypyridine, and that of Conrad and Limpach [1887] for the preparation of  $\gamma$ -chloroquinaldine from  $\gamma$ -hydroxyquinaldine, *p*-methyl- $\gamma$ -hydroxyquinaldine (12 g.), phosphorus oxychloride (12 g.) and phosphorus pentachloride (14.5 g.) were heated together in a round-bottomed flask at 130°. The preliminary rapid reaction died down almost completely at the end of 30 minutes. On cooling, the liquid set to a semi-crystalline mass. Water was now cautiously added and then a concentrated solution of sodium hydroxide until the contents of the flask were strongly alkaline. The oily mixture was then steam-distilled, when the almost colourless chloro-compound passed over and was apt to solidify in the condenser. When the distillate had stood for 24 hours, the *p*-methyl- $\gamma$ -chloroquinaldine which had separated was filtered off, dried and purified by distillation under reduced pressure. It distilled at 119°/3.5 mm., the temperature of the bath being 150–3°: yield 9.5 g., m.p. 69–70°. (Found: Cl, 18.25 %.  $C_{11}H_{10}NCl$  requires Cl, 18.54 %.)

*p*-Methyl- $\gamma$ -methoxyquinaldine (formula 4). *p*-Methyl- $\gamma$ -chloroquinaldine (15 g.) and sodium methoxide (4.5 g.) dissolved in 10 cc. methyl alcohol were heated together in a bomb tube at 140° for 2 hours. To the mixture was added excess of water with stirring, when fine crystals separated. The mixture was allowed to stand overnight and then filtered. The comparatively pure *p*-methyl- $\gamma$ -methoxyquinaldine when dry was dissolved in the minimum quantity of hot light petroleum (b.p. 110–20°). From the solution, on cooling, the quinaldine separated as a mass of colourless needles. These were dried on a porous plate: m.p. 106.5–107°, which was not raised by recrystallisation. (Found: N, 7.41 %.  $C_{12}H_{13}ON$  requires N, 7.49 %.)

*o*-Methyl- $\gamma$ -ethoxyquinaldine (formula 10). For the purpose of protecting the hydroxy-group against oxidation at a later stage of the work, it was replaced, as shown above, by a methoxy group. Substitution of the hydroxy- by an ethoxy-group would serve the same purpose, and, as such substitution can in certain cases be carried out directly by using ethyl-*p*-toluenesulphonate, the action of this reagent on *o*-methyl- $\gamma$ -hydroxyquinaldine was studied.

*o*-Methyl- $\gamma$ -hydroxyquinaldine (12.5 g.) dissolved in absolute alcohol was added to a solution of sodium (1.65 g.) in absolute alcohol. To the mixture was

then added with vigorous shaking ethyl-*p*-toluenesulphonate (14.5 g.) dissolved in a little absolute alcohol. The sodium salt of the toluenesulphonic acid began to separate within a few hours. At the end of 48 hours the mixture was boiled on a sand-bath, cooled and filtered. The excess alcohol was then distilled off from the filtrate, dilute sodium hydroxide added to the residue in the flask and the mixture shaken several times with ether. The combined ethereal extracts were then washed with water and finally with dilute hydrochloric acid. The acid extract so obtained deposited on neutralisation a colourless crystalline precipitate of *o*-methyl- $\gamma$ -ethoxyquinaldine. This was dissolved in dilute hydrochloric acid. The solution was boiled with a little charcoal and filtered. Addition of alkali reprecipitated the ethoxyquinaldine which was washed several times with distilled water. When dry, it was crystallised from a small quantity of light petroleum. The air-dried crystals melted at 55° and weighed 9.5 g. (65.5 % of theory). (Found: N, 6.90 %.  $C_{13}H_{15}ON$  requires N, 6.96 %.)

*Benzylidene-p-methyl- $\gamma$ -methoxyquinaldine* (formula 5). *p*-Methyl- $\gamma$ -methoxyquinaldine (5 g.) and benzaldehyde (4 g.) were heated together in a sealed tube for 6 hours at 175°. The slightly coloured condensation product was dissolved in alcohol, the solution boiled with charcoal and filtered. To the filtrate, while still hot, warm water was added until a cloud began to form, when it was set aside to cool. The benzylidene compound separated in the form of long straw-coloured needles; yield, 6.0 g. (81 % theory); m.p. 140–1°. (Found: N, 5.1 %.  $C_{18}H_{17}ON$  requires N, 5.09 %.) It distilled under a reduced pressure (4 mm. Hg) with the metal-bath at 140°. m.p. of the distillate 140–1°. Benzylidene-*p*-methyl- $\gamma$ -methoxyquinaldine formed a hydrochloride soluble in alcohol and precipitable therefrom by the addition of ether, m.p. 204°.

*Benzylidene-o-methyl- $\gamma$ -ethoxyquinaldine*. *o*-Methyl- $\gamma$ -ethoxyquinaldine (10 g.) and benzaldehyde (5.7 g.) were heated together in a sealed tube for 7 hours at 130–150°, and the resulting condensation product purified according to the procedure described above for the preparation of benzylidene-*p*-methyl- $\gamma$ -methoxyquinaldine. Benzylidene-*o*-methyl- $\gamma$ -ethoxyquinaldine was obtained in the form of colourless plates, which melted after repeated crystallisation from dilute alcohol at 115–6°. (Found: N, 4.78 %.  $C_{20}H_{19}ON$  requires N, 4.84 %.)

*p*-Methyl- $\gamma$ -methoxyquinoline-2-carboxylic acid (formula 6). Benzylidene-*p*-methyl- $\gamma$ -methoxyquinaldine (7 g.) and potassium permanganate (6.4 g.) were dissolved separately in minimum quantities of purified acetone. When the permanganate was completely dissolved it was added in small portions with mechanical stirring to the solution of the quinaldine compound. The mixture became warm and manganese dioxide slowly separated. When all the permanganate had been added, the mixture was set aside overnight and then filtered. The solid remaining on the Büchner funnel after being washed with acetone was extracted with 400 cc. of hot water three times. The combined aqueous extracts were cooled and neutralised with dilute acetic acid when the

*p*-methyl- $\gamma$ -methoxyquinoline-2-carboxylic acid was precipitated. Next morning the precipitate was filtered off and recrystallised from hot water. The purified product melted at 228°. (Found: N, 6.32 %.  $C_{12}H_{11}O_3N$  requires N, 6.40 %.)

*o*-Methyl- $\gamma$ -ethoxyquinolin-2-carboxylic acid. The method of preparation of this compound from benzylidene-*o*-methyl- $\gamma$ -ethoxyquinoline by oxidising it in acetone solution with potassium permanganate closely followed that described above for the preparation of *p*-methyl- $\gamma$ -methoxyquinoline-2-carboxylic acid. Owing to its greater solubility in water than the last-named compound, a slightly different procedure had to be followed to obtain it from the aqueous extracts of the precipitate which separated from the acetone solution during the initial process of oxidation. These extracts, amounting to some 1.2 l., were concentrated on the steam-bath to a small volume (75 cc.). The slightly coloured solution so obtained deposited, on cooling, the potassium salt of the *o*-methyl- $\gamma$ -ethoxyquinoline-2-carboxylic acid. The potassium salt was very soluble in water and careful neutralisation with acetic acid did not precipitate the free acid. The addition of a concentrated solution of sodium hydroxide to an aqueous solution of the potassium salt precipitated the sodium salt of the acid. The sodium salt thus obtained was purified by dissolving it in the minimum quantity of boiling water and allowing the solution to cool, when it was obtained as a mass of colourless needles. Analysis proved the compound to be pure enough for the purpose of the next experiment. (Found: N, 5.40 %.  $C_{13}H_{12}O_3NNa$  requires N, 5.52 %.)

6-Methylkynurenic acid (formula 7). *p*-Methyl- $\gamma$ -methoxyquinoline-2-carboxylic acid (3 g.) was boiled under reflux with constant-boiling hydrobromic acid (25 cc.) for 4 hours. At first the quinoline compound went into solution but after about 15 minutes the hydrobromide of the methylkynurenic acid commenced to separate. When the reaction was complete, the mixture was allowed to cool. Next morning the almost solid crystalline mass was filtered off. The crystals were then dissolved in the minimum quantity of hot sodium bicarbonate solution and filtered. Careful neutralisation of the warm solution with dilute hydrochloric acid precipitated the 6-methylkynurenic acid in the form of silky needles. The purification of the acid was accomplished by re-dissolving it in bicarbonate solution, filtering and finally precipitating it with mineral acid. The crystalline mass so obtained was washed with hot water, in which it is insoluble, until the washings were free from chloride.

*Analysis*: 4.56 mg. gave 10.860 mg.  $CO_2$ , 1.81 mg.  $H_2O$ ; C, 64.9 %; H, 4.41 %.

$C_{11}H_9O_3N$  requires C, 65.0 %; H, 4.44 %.

6-Methylkynurenic acid melted at 279° with violent decomposition. Its barium salt forms microscopic needles which are very insoluble in cold water. On long heating of the acid with acetic anhydride in an oil-bath at 140°, a reddish blue colour developed similar to that which can be obtained with kynurenic acid. The coloured compound which separated was slightly soluble

in benzene giving a blue solution. The colour faded on exposing the solution to strong sunlight. Dry 6-methylkynurenic acid heated with hydrochloric acid and potassium chlorate gave a chloro-derivative which, when treated with ammonia, turned mahogany brown, then dark green and finally deep blue (Jaffé's reaction).

The constitution of the new 6-methylkynurenic acid was established in the following manner. 2 g. of the acid in a boiling-tube were heated in a metal-bath at  $280-90^{\circ}$ . After the evolution of carbon dioxide had ceased, the tube was cooled and the semi-crystalline residue boiled with a little hydrochloric acid. The solution, after being reboiled with charcoal, was filtered and cooled, when the hydrochloride of 6-methyl- $\gamma$ -hydroxyquinoline crystallised. The crystals were dissolved in aqueous potassium carbonate, and on standing overnight 6-methyl- $\gamma$ -hydroxyquinoline (formula 8) separated out. Crystallised from hot water and dried at  $120^{\circ}$ , it melted at  $227^{\circ}$ .

1 g. of the 6-methyl- $\gamma$ -hydroxyquinoline obtained in the above manner was ground up with some pure zinc dust, and the mixture heated in a small distillation flask in a metal-bath. The oily distillate which collected in the receiver was dissolved in a little mineral acid, the solution boiled with a little charcoal, filtered and carefully neutralised with alkali. The addition of an equal volume of an aqueous solution of picric acid precipitated a brownish coloured solid which was boiled in turn with water, alcohol, ether and benzene, in all of which it is very insoluble. Dried at  $120^{\circ}$ , it melted at  $227^{\circ}$ . (M.P. of 6-methylquinoline picrate as given by Beilstein is  $229^{\circ}$ .)

*8-Methylkynurenic acid.* *o*-Methyl- $\gamma$ -ethoxyquinoline-2-carboxylic acid (3 g.) in the form of its dry sodium salt was boiled under reflux with constant-boiling hydrobromic acid (25 cc.). The solid gradually passed into solution but crystals of the hydrobromide of the 8-methylkynurenic acid did not begin to separate until some  $2\frac{1}{2}$  hours had elapsed. After 5 hours' boiling the mixture was allowed to cool and the separated crystals were filtered off next morning and dissolved in dilute sodium bicarbonate solution. This was filtered and hydrochloric acid added to the filtrate as long as any precipitate formed. After 24 hours the precipitate was crystallised from boiling water, in which it was slightly more soluble than the 6-methylkynurenic acid.

8-Methylkynurenic acid melted at  $266^{\circ}$ . It gave a barium salt which crystallised in the form of small needles slightly soluble in water. The residue obtained by heating it with hydrochloric acid and potassium chlorate to dryness did not give the Jaffé series of colour changes when treated with ammonia, nor did the free acid give a blue compound as was the result when 6-methylkynurenic acid was boiled with acetic anhydride at  $140^{\circ}$ .

*Analysis:* 4.800 mg. dried at  $100^{\circ}$  gave 10.535 mg.  $\text{CO}_2$ , 2.14 mg.  $\text{H}_2\text{O}$ .  
C, 59.9 %, H, 5.2 %.  $\text{C}_{11}\text{H}_9\text{O}_3\text{N}$ ,  $\text{H}_2\text{O}$  requires C, 59.7 %, H,  
4.98 %.

The constitution of the 8-methylkynurenic acid was established by converting it first into 8-methyl- $\gamma$ -hydroxyquinoline and then into 8-methyl-



quinoline, the picrate of which substance is already known. 8-Methylkynurenic acid (2.5 g.) was heated at 270–80°. The melt, which remained after the evolution of carbon dioxide had ceased, was cooled and boiled up with dilute hydrochloric acid and charcoal and then filtered. The filtrate, neutralised with potassium carbonate, deposited crystals of 6-methyl- $\gamma$ -hydroxyquinoline, m.p. 206°. The distillate obtained by heating this compound with zinc dust was boiled with a little hydrochloric acid and charcoal and filtered. The filtrate was carefully neutralised and treated with an aqueous solution of picric acid. The dark brownish precipitate which separated was collected, boiled with water, alcohol and benzene, and dried in the air. The picrate so obtained melted at 196°. (According to Beilstein, 8-methylquinoline picrate melts at 198°.)

#### SUMMARY.

In order that a study may be made of the behaviour in the animal organism of 6- and 8-methylkynurenic acids, these compounds have been synthesised.

The author desires to acknowledge his indebtedness to the Medical Research Council for a part-time grant. The cost of the materials for the above work was defrayed from a grant made by the Moray Fund Grants Committee, Edinburgh University.

#### REFERENCES.

- Barger and Ewins (1917). *Biochem. J.* **11**, 58.  
Besthorn (1921). *Ber. deutsch. chem. Ges.* **54**, 1330.  
Cohn (1895). *Z. physiol. Chem.* **20**, 210.  
Conrad and Limpach (1887). *Ber. deutsch. chem. Ges.* **20**, 952.  
——— (1888). *Ber. deutsch. chem. Ges.* **21**, 523.  
Ellinger and Matsuoka (1914). *Z. physiol. Chem.* **41**, 45.  
Lieben and Haitinger (1885). *Monatsh.* **6**, 315.  
Robson (1924). *J. Biol. Chem.* **62**, 495.

# CXLV. THE METABOLISM OF TRYPTOPHAN.

## III. THE MODE OF FORMATION OF KYNURENIC ACID FROM TRYPTOPHAN.

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*(Received July 3rd, 1928.)*

It has been shown by the author in a previous paper [1924] that light may possibly be thrown on the mechanism of the formation of kynurenic acid from tryptophan by feeding to an animal a tryptophan substituted in the benzene nucleus and recovering from the urine a substituted kynurenic acid. For the purpose of carrying out an investigation on these lines, the author selected *Bz-3-methyltryptophan* [Robson, 1924]. In a subsequent paper [Robson, 1928], the synthesis of the possible end-products of the metabolism of this substituted tryptophan, *i.e.* 6-methyl- and 8-methyl-kynurenic acids, has also been described. The final stage of the investigation consisted in the administration, either by feeding or by injection, of these three compounds to a dog or to a rabbit, followed by an examination of the urine for possible end-products of their metabolism.

The animal selected for the study, a rabbit, was placed on a diet of oats and water. A sample of the oats used for this purpose gave no visible reaction with the Hopkins-Cole reagent and was considered to contain very little, if any, tryptophan. After the second day, a 24 hours' sample of urine was collected and an attempt made to isolate kynurenic acid from it by Capaldi's method. The urine (75 cc.) was treated with half its volume of a solution containing barium chloride (10 %) and ammonium hydroxide (5 %). After half an hour, the voluminous precipitate which had formed was filtered off and the filtrate evaporated to half its original volume (40 cc. approx.). Careful neutralisation of the concentrate with hydrochloric acid (4 %) produced no precipitate even after 48 hours. It was inferred, therefore, that kynurenic acid was absent from the urine.

*Exp. 1<sup>1</sup>. Injection of tryptophan.* Tryptophan (1 g.) in the form of its sodium salt was injected subcutaneously into the rabbit. The urine, collected during the next 24 hours, was treated according to Capaldi's method. The kynurenic acid thus obtained weighed, on recrystallisation from alcohol, 0.13 g., and melted at 282°.

<sup>1</sup> The injections described in the present paper were carried out by Dr F. R. Curtis, to whom the author's thanks are due.

*Exp. 2. Injection of Bz-3-methyltryptophan.* Bz-3-Methyltryptophan (1 g.) in the form of its sodium salt was injected subcutaneously into the same rabbit, and the urine collected for 24 hours. This measured 70 cc. and was treated according to Capaldi's method. The concentrated urine was acidified with 4 % hydrochloric acid, and was set aside for 48 hours. No precipitate was deposited. The urine, which did not give the Hopkins-Cole reaction in the cold, was consequently freed from barium, made acid to Congo red and shaken with ether and then with ethyl acetate. Evaporation of the ethereal extract gave a very small quantity of an oily residue, reddish yellow in colour, which did not crystallise after standing 14 days in a desiccator. The ethyl acetate extract on evaporation gave a residue consisting mainly of urea; analysis of this residue failed to show the presence of any compound of indole.

A second experiment with Bz-3-methyltryptophan (0.6 g.) was carried out on the same animal and the urine collected for 48 hours. Treatment of the urine as in the previous experiments failed to reveal the presence of either of the methylkynurenic acids in the small amount of flocculent precipitate which formed on the addition of the hydrochloric acid at the final stage. The concentrated urine was then freed from barium and sulphuric acid, neutralised to litmus and shaken several times with ether. The combined ethereal extracts were washed with water and evaporated to dryness on the water-bath. The oily residue resembled in appearance that obtained in the previous experiment. It did not crystallise on long standing in the desiccator and there was not a sufficient quantity of it available for purposes of identification. It would appear from the results of these last two experiments that the Bz-3-methyltryptophan was completely burnt in the rabbit.

*Exp. 3. Injection of 6-methylkynurenic acid.* For the purpose of Exps. 3 and 4 a second rabbit was used. This animal, on the diet of oats and water, had been shown to give kynurenic acid in the urine when tryptophan was injected subcutaneously.

6-Methylkynurenic acid (0.8 g.) was injected subcutaneously and the urine collected during the next 48 hours. Submitted to Capaldi's method of analysis, it gave 0.65 g. of a substance which, on purification, melted at 277°. A melting-point determination of a mixture of the isolated substance and pure 6-methylkynurenic acid gave a reading of 277°, showing that the unknown substance was undoubtedly 6-methylkynurenic acid. It was apparent, therefore, that 6-methylkynurenic acid had passed through the organism of the animal unchanged.

*Exp. 4. Injection of 8-methylkynurenic acid.* This acid (0.9 g.) was injected into the rabbit under the same conditions as those already described. The urine, collected for 48 hours, on being treated according to Capaldi's method, gave no precipitate on standing for 2 days. The neutralised concentrate on steam-distillation gave a strongly smelling liquid, an investigation of which failed to reveal the presence of any quinoline derivative. The urine was therefore again concentrated, acidified and shaken with ether. The ethereal extracts on evapo-

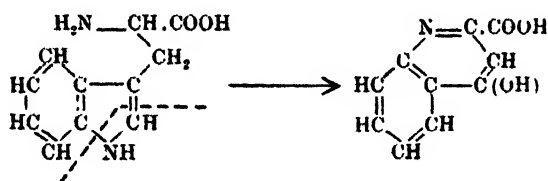
ration gave a small quantity of a reddish oil which did not crystallise on long standing in the desiccator.

A second experiment with 1.2 g. of 8-methylkynurenic acid was carried out. Analysis of the urine collected failed to indicate the presence either of the methylkynurenic acid which had been fed, or any of its possible derivatives. It would appear, therefore, that 8-methylkynurenic acid is completely burnt in the animal organism.

#### DISCUSSION.

From the results of the feeding experiments described above it is seen that, whereas 6-methylkynurenic acid was recovered almost quantitatively from the urine of the animal, no determinable end-products were obtained in the case of *Bz*-3-methyltryptophan and of 8-methylkynurenic acid. In one sense, since no derivative was isolated from the urine when *Bz*-3-methyltryptophan was injected, the results of the experiments with this compound can scarcely be regarded as wholly satisfactory.

On the other hand, however, if reference is made to the diagram [Robson, 1928] illustrating the possible ways in which kynurenic acid may be formed from tryptophan it will be seen that, according to Theory II, *Bz*-3-methyltryptophan gives rise to 8-methylkynurenic acid. The fact that both *Bz*-3-methyltryptophan and 8-methylkynurenic acid are completely burnt in the animal organism strongly suggests that the latter compound is an intermediate metabolite of the former. Such a transformation would involve the elimination of the pyrrole ring according to the following diagram:



Evidence in the literature in favour of the transformation occurring in this way is not entirely lacking. For example, Ellinger and Matsuoka [1914] and Barger and Ewins [1917] on feeding *Pr*-2-methyltryptophan to an animal failed to detect the occurrence in the urine of a corresponding methylated kynurenic acid, and as a result the latter workers concluded that such a transformation did not take place because the pyrrole ring had been rendered more stable by the presence of the methyl group in the  $\alpha$ -position. Again, such nitrogen-ring formation by the utilisation of the side chain of an amino-acid—which the above formation of kynurenic acid would demand—is not without precedent in the literature. For instance, Raper [1927] has definitely shown that tyrosine may give rise, under the action of tyrosinase, to 5 : 6-dihydroxyindole and 5 : 6-dihydroxyindole-2-carboxylic acid, where the amino-nitrogen is the precursor of the nitrogen of the pyrrole ring.

It would appear, therefore, that the results of the feeding experiments with *Bz-3-methyltryptophan*, 6-methylkynurenic acid and 8-methylkynurenic acid form additional evidence in favour of the theory, originally brought forward by Ellinger, that the formation of kynurenic acid in the animal organism involves the elimination of the pyrrole ring of the tryptophan molecule.

#### SUMMARY.

*Bz-3-methyltryptophan*, when injected into a rabbit, is completely burnt. In view of the fact that a like fate is suffered by 8-methylkynurenic acid in the organism of this animal, whilst 6-methylkynurenic acid can be recovered from the urine of the animal almost quantitatively, it is suggested that in the formation of kynurenic acid from tryptophan the pyrrole-nitrogen of the latter compound is eliminated and the side chain with its amino-nitrogen forms the new pyridine ring.

The author desires to acknowledge the receipt of a part-time grant from the Medical Research Council.

#### REFERENCES.

- Barger and Ewins (1917). *Biochem. J.* **11**, 58.  
Ellinger and Matsuoka (1914). *Z. physiol. Chem.* **41**, 325.  
Raper (1927). *Biochem. J.* **21**, 89.  
Robson (1924). *J. Biol. Chem.* **62**, 495.  
— (1928). *Biochem. J.* **22**, 1157.

# CXLVI. THE DETERMINATION OF PYRUVIC ACID.

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*(Received May 25th, 1928.)*

THE methods employed for the estimation of pyruvic acid are largely based upon the reaction of its carbonyl group with phenylhydrazine. Its estimation in complex biological fluids where there are other compounds which react likewise is therefore difficult and, particularly when pyruvic acid exists in small quantities amidst these compounds, the estimation is unreliable even when controls are run. This difficulty was greatly felt in some of our investigations, and it was thought worth while to study some of the existing methods which could be satisfactorily employed as such or modified for our purpose.

The production of an intense blue colour by sodium nitroprusside and ammonia, especially in the presence of a little acetic acid, has been claimed to be specific for pyruvic acid and unaffected by acetaldehyde.

The precipitation of pyruvic acid as hydrazone by excess of phenylhydrazine followed by the determination of the excess phenylhydrazine has been the subject of considerable study [Smedley-MacLean, 1913; Simon and Piaux, 1924].

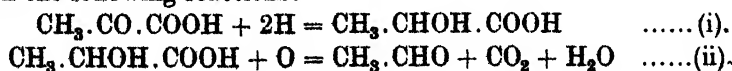
The reduction of pyruvic to lactic acid by zinc and hydrochloric acid and the subsequent estimation of lactic acid according to the method of Fürth and Charnass has been adopted by Lieben [1923].

The oxidation of pyruvic to acetic acid by a slight excess of hydrogen peroxide has been shown to be quantitative by Holleman [1904], and potassium dichromate has been employed similarly and the excess dichromate estimated.

As it is not the purpose of this paper to give the results of the comparative study of these methods, it will be sufficient to say that none of them as such was found suitable in our experiments, and all required some modifications before they could be employed.

The necessary conditions for a method which would be suitable to our requirements were that it should be applicable to small quantities of the acid, usually between 0.5 and 15 mg., and in solutions of very low concentration, i.e. 0.1 % to 0.05 %.

The method finally developed is a modification of Lieben's technique, and is based upon the following reactions:



The reduction of pyruvic to lactic acid by zinc and hydrochloric acid has been shown to be quantitative by Lieben. The lactic acid was estimated according to Fürth and Charnass. A micro-adaptation of Fürth and Charnass's method, with small quantities of pyruvic acid reduced by zinc and hydrochloric acid, gave very irregular results, due probably to very slight but irregular decomposition of pyruvic acid when boiled for a long time with 10 % HCl. With higher concentrations of pyruvic acid the results are of the same degree of accuracy as those of Lieben, and, with an empirical factor for lactic acid, its equivalent of pyruvic acid can be accurately calculated. Some of the results obtained according to the micro-adaptation of Fürth and Charnass's method of oxidation, and for larger amounts of acid according to the original method, are given in Tables I and II respectively. In all these cases, however, the titration of bound aldehyde was made according to Clausen [1922].

Table I.

Different amounts of pyruvic acid were reduced by boiling with 50 cc. of 10 % HCl and 0.5 to 0.75 g. of zinc for 2½ hours.

Pyruvic acid taken (mg.)	0.68	1.36	1.36	2.04	2.04	3.40	3.40	2.72
Found (%)	79.4	85.3	81.6	85.3	82.9	86.5	82.9	83.1
Pyruvic acid taken (mg.)	2.72	6.80	6.80	10.20	10.20	13.60	13.60	
Found (%)	84.6	85.5	82.0	86.2	88.8	89.2	87.1	

According to the same method Clausen finds with lactic acid a yield on an average of approximately 92 %, about the same as that found for larger quantities. For comparison some results of the estimation of larger quantities of pyruvic acid according to the original method of Fürth and Charnass are given in Table II.

Table II.

Different amounts of pyruvic acid were reduced by boiling with 100 cc. of 10 % HCl and 2 g. of zinc for 2½ hours.

Pyruvic acid taken (mg.)	12.14	12.14	24.28	24.28	36.42	36.42	48.56
Found (%)	86.9	85.8	86.7	90.2	88.3	87.9	88.5
Pyruvic acid taken (mg.)	48.56	60.7	60.7	121.4	121.4	182.1	182.1
Found (%)	89.9	88.4	90.1	89.56	91.6	89.6	92.2

The values for pyruvic acid computed according to the empirical factor of Fürth and Charnass give results accurate to within about  $\pm 3$  %.

The irregular results obtained when small amounts of pyruvic acid are estimated were at first supposed to be due to the length of time during which they were in contact with boiling acid, and unsuccessful attempts were made to find a time during which a maximum and constant yield was obtained.

In view of this irregularity, reduction at low temperatures was tried and the use of a zinc-copper couple in sulphuric acid solution was found satisfactory. The oxidation of lactic acid was carried out by a slight modification of a recent method due to Friedmann *et al.* [1927]. In this case the yield was lower than with other methods (about 80 %) but it was quite constant within a large range of pyruvic acid concentrations.

The apparatus used by us is shown in Fig. 1 and is much simpler than that of Friedmann. The transference of aldehyde to the receiver is facilitated by passing carbon dioxide from a cylinder through the apparatus. The gas is passed through a long column of saturated sodium bisulphite solution and then through water, which is frequently changed, before it enters the reaction vessel. The yield of aldehyde has been found to depend upon the rate of aeration also, and this can be effectively controlled.

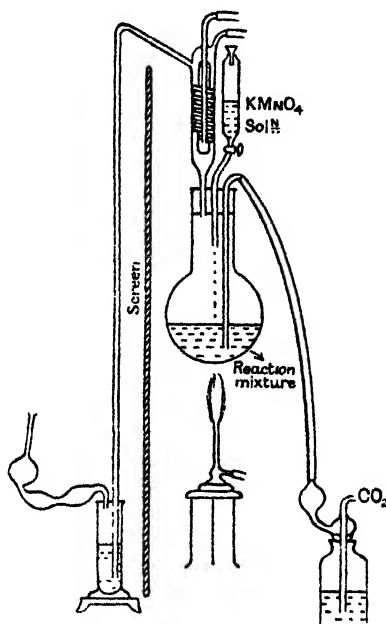


Fig. 1.

#### THE ESTIMATION OF PYRUVIC ACID IN PURE SOLUTIONS.

1 to 5 cc. of solution containing 0.25 to 15 mg. of pure pyruvic acid are treated with 50 cc. of 17.5 %  $\text{H}_2\text{SO}_4$  and 0.5 to 1 g. zinc together with 1 cc. of 10 % copper sulphate solution. After an hour the solution is filtered into the reaction flask (a 500 cc. Kjeldahl flask) and neutralised slowly with 60 %  $\text{NaOH}$  added drop by drop, dimethylaminoazobenzene being the indicator. 10 cc. of 10  $N$   $\text{H}_2\text{SO}_4$  containing 0.1  $N$   $\text{MnSO}_4$  are then added and the oxidation is carried out with 0.01 or 0.005  $N$  permanganate, the bound aldehyde being then titrated according to Clausen's method. The use of hydroxylamine to catch the acetaldehyde, as suggested by Leone and Tafuri [1925], was tried, but the end-point was not very sharp, and in all further work Clausen's method was adopted. Some of the results of estimations of pyruvic acid in pure solution with different quantities of the acid are given in Table III. A few experiments were also conducted in which the oxidation of the reduced



pyruvic acid was carried out with sulphuric acid. The yield of aldehyde obtained when 50 % sulphuric acid at 140° was used according to Clausen showed that more drastic treatment was required, namely increase of the temperature to 160°–170° and the use of 60 % acid, as had been found by Hill, Long and Lupton [1924] and Ronzoni and Laurence [1927]. Some of the results obtained are given in Table IV.

Table III. *Analyses of pyruvic acid in pure solutions.*

Pyruvic acid taken (mg.)	16.36	16.36	13.62	13.62	8.18	8.18	6.10	6.10
Found (%)*	99.6	99.4	101.9	97.8	99.3	99.3	100.6	100.6
Pyruvic acid used (mg.)	3.44	2.44	2.44	1.22	1.22	0.61	0.61	0.305
Found (%)*	97.7	97.6	97.6	102.3	98.4	103.2	100.0	100.0

\* Using factor (see p. 1173).

Table IV. *Comparison of the permanganate and sulphuric acid methods of oxidation.*

Pyruvic acid taken (mg.)	8.43	8.43	5.06	3.37	0.61	0.305	1.69
Permanganate method, found (%)	80.4	82.3	80.3	78.9	80.3	82.6	76.3
Sulphuric acid method, found (%)	80.9	80.7	81.0	82.8	78.7	78.7	78.0

The results of the estimation of pyruvic acid in pure solution according to both the methods of oxidation, showed that there is a yield of 80 % aldehyde, constant over large variations in the quantities of pyruvic acid used. This does not appear to be due to a factor in the oxidation of lactic acid since pure zinc lactate solutions could be determined by the same method with an accuracy of 96.2 to 97.9 %.

The actual losses of pyruvic acid appear to occur during the initial reduction process, probably due to the formation, along with lactic acid, of products which do not yield bisulphite-binding compounds during the subsequent oxidation. Ronzoni and Laurence [1927], in connection with their experiments on the loss of lactic acid in solution, drew attention to an early observation of Dakin, who found that lactic acid solutions on standing yielded methylglyoxal. They moreover found that a solution of zinc lactate on standing for several days gave smaller and smaller yields of aldehyde day by day until after 4 days the yield stood constant at about 84 % for several weeks. This fall was noticed even when the solution was sterile and in the presence of mercuric chloride. We ourselves have observed such a loss of aldehyde, but the fall, though considerable, was not so rapid as that found by Ronzoni and Laurence. It was also found that zinc lactate solution, treated in the same way as pyruvic acid in our experiments, gave lower yields of aldehyde.

Another source of loss of lactic acid before oxidation appears to be due to the fact that it is kept in contact with sulphuric acid, and this loss increases with the time of contact. 23.3 mg. of zinc lactate were made up to 100 cc. with 20 % sulphuric acid. 20 cc. of this solution were taken at intervals of 1½ hours and the lactic acid was estimated. The results are given in Table V.

Table V.

Time during which lactic acid was in contact with sulphuric acid (hrs.)	0	1½	3	4½	6
Aldehyde yield (%)	97.6	96.5	95.4	94.2	93.1

Taking all these observations into consideration, we are led to imagine that the small yield of aldehyde in our case may be due to the same cause as that which lessened the yield of aldehyde with lactic acid solution on standing. The possibility exists of the formation of methylglyoxal from pyruvic acid if the carboxyl group is attacked, along with the carbonyl group, through the vigorous reducing action of the zinc-copper couple, although the formation of methylglyoxal is not known to occur from reduction of pyruvic acid in practice.

Since in the estimation of pyruvic acid by this method the errors due to reduction or oxidation are quite regular over a large range of concentration of pyruvic acid, the particular drawback of a small yield of aldehyde and the consequent necessity of using a large factor to compute the actual amount, cannot materially diminish the value of the method for the estimation of small quantities of pyruvic acid. This is shown by calculating the probable error of a single determination by the formula

$$e = \pm 0.6745 \sqrt{\frac{d_1^2 + d_2^2 + \dots + d_n^2}{n-1}},$$

where  $d$  represents the deviation of an observation from the mean of the series and  $n$  the number of observations. The probable error of the mean ( $e_m$ ) is given by the equation

$$e_m = \frac{e}{\sqrt{n}}.$$

The probable error from a series of 15 determinations on the same quantity of pyruvic acid is shown in Table VI, and also the probable error of the mean.

Table VI.

10.18 mg. of pyruvic acid were reduced by 40 cc. of 17.5 %  $\text{H}_2\text{SO}_4$  plus 0.75 g. Zn and a trace of copper sulphate.

Amount of acid taken (mg.)	Amount found (mg.)	Deviation from the mean ( $d$ )	$d^2$
10.18	8.10	-0.04	0.0016
	8.18	+0.04	0.0016
	8.04	-0.10	0.0100
	8.10	-0.04	0.0016
	8.03	-0.11	0.0121
	7.92	-0.22	0.0484
	8.27	+0.13	0.0169
	7.90	-0.24	0.0576
	8.06	-0.08	0.0064
	7.95	-0.19	0.0361
	8.14	+0.00	0.0000
	8.00	-0.14	0.0196
	8.29	+0.15	0.0225
	8.06	-0.08	0.0064
	8.16	+0.02	0.0004

Mean = 8.14

$\Sigma d^2 = 0.2412$

therefore  $e = 0.0885$

and the error of mean ( $e_m$ ) = 0.0237.

The probable error of a single determination is, therefore, about 1.1 %. The mean of this series is very nearly the same as that obtained from the determination with different quantities of pyruvic acid (Table III).

Thus, if an empirical factor covering the regular error be taken, the exact amount of pyruvic acid can be computed. This factor works out to about 20 %, *i.e.* 1 cc. *N*/10 iodine represents 5.5 mg. pyruvic acid. The values calculated on this empirical basis for various quantities of pyruvic acid have been given in Table III.

#### APPLICATION OF THE PROPOSED METHOD TO BIOLOGICAL FLUIDS.

The question of the specificity of the reactions upon which the proposed method of estimation is based arises when the method is applied to biological fluids. It will be seen that not only hydroxy-acids, carbohydrates and acetone but also lactic acid and acetaldehyde interfere with the determination and must be removed from the solution before pyruvic acid is estimated. The separation of pyruvic acid from other bisulphite-binding compounds is not easy, and the results of titration cannot be taken as specific unless acetaldehyde from pyruvic acid only be taken as a measure of that acid. During the usual processes for the removal of carbohydrates or proteins from biological materials, these interfering agents are not removed. It is found that pyruvic acid bound by sodium bisulphite is not extractable by ether, whereas lactic acid and other hydroxy-acids are easily extracted. Therefore the pyruvic acid may be extracted along with lactic acid and other interfering substances by a preliminary ether extraction which separates them from carbohydrates also. The ether extract with a small quantity of sodium bisulphite solution is again extracted with ether, when the pyruvic acid, which is combined with bisulphite, is separated from lactic acid,  $\beta$ -hydroxybutyric acid and other interfering substances.

Thus, since ether extraction was used for the separation of pyruvic acid from carbohydrates and lactic acid, there was no necessity to adopt Van Slyke's method [1917] for the removal of carbohydrates from solution. It was moreover found that considerable amounts of pyruvic acid are lost in that process.

We had therefore to study only the separation of proteins from dilute pyruvic acid solution. Experiments were carried out with caseinogen and haemoglobin solutions to which small quantities of pyruvic acid were added. In the case of body fluids, it is found that the method of Folin and Wu [1919] can be employed. For the separation of proteins from yeast culture solutions, precipitation by alcohol is sometimes preferable. The solution is rendered faintly acidic and 15 to 20 times its volume of 99 % alcohol added. The solution is kept overnight, filtered, and the precipitate washed with 98 % alcohol.

An aliquot portion of the solution after the precipitation of proteins, either by the method of Folin and Wu or by the use of alcohol, is neutralised and

evaporated under reduced pressure at 40° to 50° almost to dryness. The residue is then transferred with a small quantity of saturated ammonium sulphate solution to the ether extractor, the solution rendered acid to methyl red, and the pyruvic acid extracted. The ether is evaporated and the pyruvic acid is bound by sodium bisulphite, and the solution is extracted again with ether to remove lactic acid, etc. It is estimated as before and the exact amount calculated by means of the empirical factor.

Table VII. *Recovery of pyruvic acid from protein solutions.*

Pyruvic acid added (mg.)	Protein solution	Method of precipitation	Pyruvic acid recovered (%)
4.61	2 % haemoglobin	Folin and Wu	99.2
4.61	5 % "	" "	98.9
4.61	2 % "	Alcohol "	99.35
4.61	5 % "	" "	98.9
7.69	3 % "	Folin and Wu	99.45
7.69	3 % "	Alcohol	100.1
15.37	5 % "	Folin and Wu	99.48
15.37	5 % "	Alcohol	97.8
3.05	2 % caseinogen	Folin and Wu	98.4
3.05	2 % "	Alcohol	97.7
6.10	3 % "	Folin and Wu	98.5
6.10	3 % "	Alcohol	100.2
15.25	3 % "	Folin and Wu	99.2
15.25	3 % "	Alcohol	98.3
15.25	5 % "	Folin and Wu	97.9
15.25	5 % "	Alcohol	97.6

From such dilute solutions of pyruvic acid a recovery of 97 % is therefore possible after protein precipitation.

The estimation of pyruvic acid in biological fluids may therefore be carried out as follows. The solution, which should contain not more than 15 mg. of pyruvic acid during its reduction to lactic acid, is taken and the proteins separated by either of the processes detailed above. Suppose the solution contains 0.05 %, about 2 to 5 cc. of this is used for protein precipitation and the whole of the filtrate is taken up for subsequent processes. The filtrate is rendered neutral to litmus and evaporated under diminished pressure at 40° to 50°. In this process much of the preformed acetone, acetaldehyde or other volatile compounds, escapes. The substance is then transferred to a Clausen or Meyerhof extractor with a small quantity of saturated ammonium sulphate solution, rendered slightly acid and extracted with ether to separate the carbohydrates from the ether-extractable substances. The ether should have been distilled over bisulphite, as it sometimes contains bisulphite-binding compounds as impurities. The ether extract is evaporated to dryness and shaken up with a quantity of sodium bisulphite three or four times in excess of the expected amount of pyruvic acid. It is then transferred again to the ether extractor to separate pyruvic acid from lactic acid,  $\beta$ -hydroxybutyric acid, phenols, etc. The residue is transferred to a 100 cc. flask and the pyruvic acid reduced by sulphuric acid and zinc with a trace of copper. The lactic acid is estimated as already described.

The errors introduced by the presence of acetone bodies is considerably diminished by the two evaporations. But even then the error due to acetone formed during oxidation, or held tenaciously, can be determined after the removal of aldehyde by Schaffer's method [1908], as adopted by Clausen as a correction for his lactic acid determination. The titre from this distillation is subtracted from the previous titre, and after proper corrections are applied the pyruvic acid is calculated using as factor 1 cc. of  $N/100$  iodine = 0.55 mg. pyruvic acid. In many cases, except where the determination is carried out with yeast culture solutions, this determination of acetone bodies after final iodine titration is unnecessary, as the error is small. The results of the recovery of pyruvic acid added in small quantities to biological fluids, such as the body fluids of the lac insect, sheep's blood and yeast culture solutions, are given in Table VIII. The probable error of a single determination in a series, calculated from a number of experiments as before, is shown in Table IX.

Table VIII. *Recovery of pyruvic acid added to biological fluids.*

Medium	Amount added (mg.)	Iodine titre corrected for blank and titre (cc.)	Control (cc.)	Amount recovered using the empirical factor	Error (%)
Sheep's blood, 5 cc.	1.51	5.5 (0.005 $N$ )	0.10?	1.49	-1.3
	3.02	11.3 (0.005 $N$ )	0.10?	3.08	+2.0
	6.04	11.15 (0.01 $N$ )	0.10?	6.08	+0.7
Yeast culture solution, 5 cc.	1.51	5.65 (0.005 $N$ )	0.25	1.49	-1.3
	3.02	11.12 (0.005 $N$ )	0.25	2.99	-1.0
	6.04	10.96 (0.01 $N$ )	0.25	5.89	-2.5
Body fluid of lac insect, 5 cc.	1.51	5.76 (0.005 $N$ )	0.20	1.53	+1.3
	3.02	11.02 (0.005 $N$ )	0.20	2.98	-1.5
	6.04	10.86 (0.01 $N$ )	0.20	5.87	-2.9

Table IX.

4.48 mg. of pyruvic acid were added to 5 cc. of the body fluid of the lac insect.

Exp. no.	Amount of pyruvic acid calculated (mg.)	$d$	$d^2$
1	4.44	+0.05	0.0025
2	4.30	-0.09	0.0081
3	4.38	-0.01	0.0001
4	4.51	+0.12	0.0144
5	4.35	-0.04	0.0016
6	4.32	-0.07	0.0049
7	4.27	-0.12	0.0144
8	4.56	+0.17	0.0289
9	4.46	+0.07	0.0049
10	4.36	-0.03	0.0009

Mean = 4.39.

Probable error  $e = 0.063$ ; i.e. about 1.4 %.

#### SUMMARY.

1. A new method for the estimation of pyruvic acid in small quantities has been described which is claimed to be more specific than the existing methods when applied to biological fluids.

2. The probable error of a single determination has been determined.

Our grateful thanks are due to Prof. R. V. Norris, for the keen interest he has shown in the work.

## REFERENCES.

- Clausen (1922). *J. Biol. Chem.* **52**, 263.  
Folin and Wu (1919). *J. Biol. Chem.* **38**, 81.  
Friedmann, Cotonio and Schaffer (1927). *J. Biol. Chem.* **73**, 335.  
Hill, Long and Lupton (1924). *Proc. Roy. Soc. Lond. B*, **96**, 455.  
Holleman (1904). *Rec. Trav. Chim.* **23**, 147.  
Leone and Tafuri (1925). *Ann. Chim. Applic.* **15**, 206.  
Lieben (1923). *Biochem. Z.* **135**, 240.  
Ronconi and Laurence (1927). *J. Biol. Chem.* **74**, 363.  
Schaffer (1908). *J. Biol. Chem.* **5**, 211.  
Simon and Piaux (1924). *Bull. Soc. Chim. Biol.* **6**, 477.  
Smedley-MacLean (1913). *Biochem. J.* **7**, 611.  
Van Slyke (1917). *J. Biol. Chem.* **32**, 455.

## CXLVII. THE SPECIFICITY OF THE DEHYDRASES.

### THE SEPARATION OF THE CITRIC ACID DEHYDRASE FROM LIVER AND OF THE LACTIC ACID DEHYDRASE FROM YEAST.

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*(Received June 26th, 1928.)*

SINCE Thunberg [1920] showed by means of the methylene blue technique that tissues could oxidise a variety of substances, there has been a question as to the specificity of the systems involved. It seemed unnecessarily complicated to assume a separate enzyme for each acid oxidised. Yet a high degree of specificity is the characteristic of most enzymes, and it is therefore unjustified, until definite proof is available, to assume that one system is responsible for the oxidation of, say, citric and lactic acids. Thunberg believed that each substance oxidised had a specific enzyme, but this "is rather unconvincingly inferred from their unequal resistances to thermal changes" [Dakin, 1921].

In addition to this evidence, there is the extraction from muscle of the succinic acid dehydrase [Ohlsson, 1921], which has recently been separated from fumarase [Alwall, 1928]. Wishart [1923] extracted the succinic acid enzyme from liver, and Stephenson [1928] extracted the lactic acid dehydrase from bacteria. It has been shown [Bernheim, 1928] that the aldehyde oxidase in potato can reduce methylene blue and this enzyme can thus be classed as a dehydrase, as can the xanthine oxidase. Although the latter enzyme has been obtained chiefly from milk it is also possible to separate it from liver [Bernheim and Dixon, 1928] and from spleen [Morgan, 1926].

Investigation of the specificities of these four enzymes has shown that their action is almost entirely confined to their respective substrates. In the present paper the separation and properties of two further dehydrases are described, and these have also proved to be highly specific.

#### THE CITRIC ACID DEHYDRASE.

The oxidation of citric acid in the body has not been extensively studied. Battelli and Stern [1911] showed that the addition of citric acid to the liver increased the carbon dioxide output, and that this increase occurred just the same when the liver and the citrate were incubated *in vacuo* as when oxygen

was present. Meyerhof [1919] did a few experiments on the oxidation of citric acid by frog's muscle. The addition of methylene blue to the tissue and fumaric acid (which he regards as belonging to the same class as citric acid in respect to its oxidation by muscle) causes as much as a 30 % increase in the oxygen uptake. This may mean two things: (1) that the system reduces methylene blue more readily than it takes up oxygen, and the reoxidation of the methylene white gives the observed increase in the oxygen uptake, or (2) the system is unable to use oxygen directly but reduces a hydrogen acceptor in the tissue which is then reoxidised by oxygen. The increase of the oxygen uptake on addition of methylene blue would be due to a greater concentration of hydrogen acceptor being available.

The ability to utilise citric acid seems to be greater in herbivora than in carnivora, for Salant and Wise [1907] have shown that injected citrate disappears more quickly from the blood of the former than from that of the latter. Finally Amberg and McClure [1924] showed that about 100 mg. of citric acid were excreted in the urine every 24 hours.

In the following an enzyme has been separated from liver which will reduce methylene blue in the presence of sodium citrate but will not take up oxygen in a Barcroft apparatus. This is also true of the lactic acid dehydrase separated from *B. coli* by Stephenson, and from yeast in work described below. The product which citric acid yields has not been identified, but in the case of the lactic acid enzyme pyruvic acid is formed and has been definitely identified, so that there is no doubt that experiments carried out with methylene blue represent a true oxidation. In the intact animal, some of the citric acid may be directly oxidised to carbon dioxide and water, but there is some evidence that a part may be converted into sugars [Greenwald, 1915].

#### *Preparation of the enzyme.*

One pound of fresh liver, of pig, ox or sheep, is minced as finely as possible and treated four times with 300 cc. of acetone, each 300 cc. being filtered off with suction before the next is added. The resulting preparation is put in a desiccator and the acetone evaporated off *in vacuo*. The dried powder will then keep for several weeks in the air. To obtain a solution of the citric acid dehydrase, 30 g. of acetone liver are ground in a mortar with 100 cc. of water, and allowed to stand 2 to 3 hours at room temperature with intermittent grinding. The mixture is then squeezed through muslin and centrifuged, and the clear red solution is placed in a collodion sac and dialysed against distilled water for 6 or 7 hours. As the salts dialyse out a precipitate appears which after the dialysis is finished is centrifuged off. The clear solution containing haemoglobin is then used for the experiments.

To get rid of the haemoglobin the solution is half saturated with ammonium sulphate and the precipitate filtered off. The filtrate contains the haemoglobin but not the enzyme. The precipitate is washed with half saturated ammonium sulphate and filtered again. It is dried on the filter paper in a desiccator.



The dry powder thus obtained is dissolved in distilled water giving a clear light brown solution. The colour is due to traces of methaemoglobin that have been adsorbed on to the precipitate. The filtration in this process is slow, and the resulting enzyme has lost some of its activity. Therefore unless otherwise specified the solution containing the haemoglobin is used.

This solution will keep for several days in the ice chest. During this time a small amount of precipitate may settle out which is then centrifuged off without affecting the activity of the enzyme. This may also occur occasionally during an experiment when the solution is in a vacuum tube. The precipitate is probably a remnant of the protein which settles out during dialysis. The enzyme can be obtained in a powdered form from the haemoglobin solution by fully saturating with ammonium sulphate. The filtration of the resulting precipitate is very rapid and the precipitate can be dried in a desiccator. The powder gives a clear red solution with approximately the original activity of the enzyme. The activity of any given solution, however, depends on the liver used and the method of preparing the acetone liver. Quick treatment with acetone and thorough drying *in vacuo* is desirable.

If *M/15* disodium hydrogen phosphate is used instead of water, the enzyme comes into solution in the same way but is accompanied by the succinic and xanthine dehydrases. None of the other common dehydrases is, however, present. When the clear centrifugate is dialysed a precipitate appears which is centrifuged off. The succinoxidase can thus be got into a clear solution whereas Ohlsson's preparation is turbid. The enzyme is not very active, and more work is necessary on the details of its preparation.

Other tissues have been tried as well as liver with similar results although the enzymes extracted were not very active. Ordinary muscle and the liver of embryo rats behave in the same way when treated with acetone and extracted with water or alkaline phosphate solution.

#### *The specificity and properties of the enzyme.*

*Oxygen uptake of the enzyme.* 3 cc. of a clear solution, an amount which will reduce 1 cc. of 1 : 5000 methylene blue solution in 20-30 minutes, were placed in a Barcroft apparatus and the oxygen uptake measured after 0.1 cc. of molar sodium citrate solution was added. No appreciable uptake occurred in 5 hours. The enzyme was then tested for its ability to reduce methylene blue, and it was found that the velocity of reduction had decreased, indicating a partial destruction of the enzyme due probably to the shaking in the air. This explains why the theoretical amount of oxygen is not taken up when methylene blue is added to the solution in the Barcroft apparatus.

The inability to take up oxygen by itself seems to be the property of the enzyme in the liver, at any rate under the conditions of the experiment. The acetone liver and the fresh liver from which the solution was made both reduce methylene blue more rapidly in the presence of citrate. Neither the acetone liver nor the fresh liver will take up more oxygen in the presence of citrate

than in its absence. The reason for this is not clear. Further work is being done on the general question of the oxygen uptake of the dehydrases.

Inorganic nitrate and *m*-dinitrobenzene [Lipschitz, 1921] were tried as possible hydrogen acceptors. The former was not reduced but the latter was. 0.1 g. of *m*-dinitrobenzene was added to solutions of enzyme with and without citrate. After incubation *in vacuo* at 37° for 2 hours, a few drops of soda were added to each. A deep purplish red colour developed immediately in the tube containing the citrate while a much lighter colour developed slowly in the control. Because of the original colour of the solution it was impossible to detect the yellow colour which develops before adding the soda.

The clear solution of the enzyme is specific for citric acid. None of the other possible hydrogen donors tried was oxidised. They included the following acids as sodium salts: succinic, malic, fumaric, lactic, tartaric, formic, glutamic, maleic, acetic,  $\alpha$ -hydroxybutyric, oxalic, saccharic, and acetaldehyde. These were made up in 5 % solutions of which 0.1 cc. was used for each experiment. Thunberg vacuum tubes were used containing 3 cc. of the solution to be tested, 2 cc. of buffer, and 1 cc. of 1 : 5000 methylene blue. The time for complete reduction of the dye at 37° was noted.

The effect of aconitic acid was then tried. This is the unsaturated acid corresponding to citric acid. It is conceivable that under the influence of the enzyme it might add water to form citric acid which then could be utilised to reduce the methylene blue. Incubation of the enzyme with sodium aconitate showed that this was not the case. But because of the similarity of structure with citric acid, aconitic acid inhibits the reduction of methylene blue by the citric acid-enzyme system. The curve obtained seems to indicate that the aconitic acid is adsorbed on the enzyme surface so that part of the surface is unavailable for the citric acid. It is probable that increasing concentrations of aconitic acid act like increasing concentrations of citric acid, namely, in keeping the methylene blue from being accessible to the active centres. This will be discussed more fully below.

The effect of aconitic acid is specific, for the additions of acids like succinic, lactic or tartaric do not increase the reduction time at all. Fig. 1 shows the effect of varying amounts of aconitic acid on the reduction of methylene blue by the enzyme-citric acid system.

The reduction time of methylene blue was obtained when varying amounts of citric acid were present. In this experiment the haemoglobin-free solution was used. As is shown in Fig. 2 the activity, *i.e.* the reciprocal of the reduction time multiplied by 100, increases rapidly with increasing concentrations of citric acid and then falls off. The activity drops sharply from 0.01 cc. of 3*N* citric acid to 0.001 cc., *i.e.* *N*/200 and *N*/2000 respectively. *N*/2000 represents about twice the theoretical quantity of citric acid necessary to reduce the methylene blue present as calculated for two hydrogen atoms for every citric acid molecule. The rate thus falls off at a comparatively high concentration, namely, *N*/200.

The significance of this maximum is probably associated with the saturation of the enzyme surface with an amount of citric acid which enables an easy access of methylene blue, and yet is concentrated enough to ensure a quick

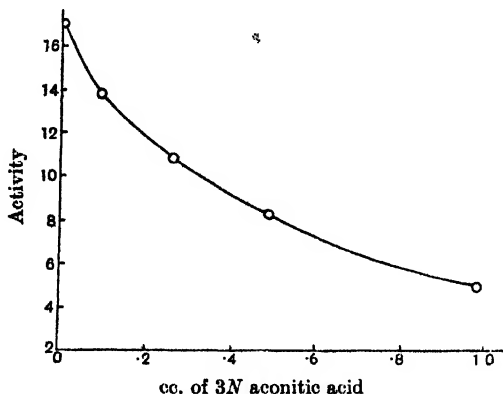


Fig. 1. Effect of aconitic acid on the reduction of methylene blue by citric acid.

reduction. When more citric acid is present the surface becomes supersaturated so that the methylene blue is no longer easily available. The inhibition is not a salt effect for equal amounts of *N* lactic acid or succinic acid do not inhibit. This inhibition at high concentrations of substrate has also been observed with the xanthine oxidase [Dixon and Thurlow, 1924]. In the following curve the concentration of citric acid is given in cc. per 6 cc.

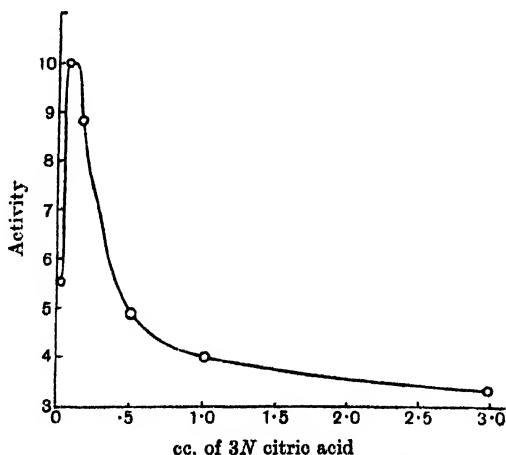


Fig. 2. Effect of varying concentrations of citric acid on the reduction of methylene blue.

## THE LACTIC ACID DEHYDRASE.

Lactic acid is not considered to be an intermediate product in the fermentation of sugars by yeast. It can, however, always be detected in yeast by extracting with acids or alkalis and applying the thiophen test to the extract. It must, therefore, play some part in the general metabolism. What its function is, is still doubtful. It is oxidised with difficulty. Fürth and Lieben [1922, 1, 2] found that a vigorous stream of oxygen through the yeast suspension was required before any appreciable oxidation occurred. They measured the oxidation by the increase of carbon dioxide produced when lactic acid was added to the suspension. The carbon dioxide produced was not however equivalent to the lactic acid present. They found that the lactic acid went partly into an ether-soluble substance and partly into the cell protoplasm. Hoffert [1926] showed that the lactic acid was not converted into unhydrolysable sugars or sugar-containing proteins. These workers have shown pyruvic acid to be present after allowing yeast to act on lactic acid. Harden and Norris [1915] showed that dried yeast plus lactic acid will readily reduce methylene blue and that pyruvic acid is quantitatively formed. This has been confirmed using untreated yeast and zymine (acetone yeast) by the following work, and is interesting in view of the observations of Fürth and Lieben that the addition of methylene blue made no appreciable difference to the amount of carbon dioxide arising from the oxidation of the lactic acid.

It is easy to show that whole yeast as well as dried yeast and zymine will reduce methylene blue more rapidly when lactic acid is present than without it, and that pyruvic acid is formed. It might therefore be expected that the pyruvic acid formed by the addition of methylene blue to the yeast lactic acid mixture would become decarboxylated and cause a rise in the carbon dioxide output. That Fürth and Lieben did not find this to be the case indicates the inability of the yeast to decarboxylate all the pyruvic acid formed.

But the following anomaly remains. Yeast contains a large amount of the lactic acid dehydrase as measured by the ability of yeast, dried yeast, or zymine to reduce methylene blue in the presence of lactic acid. For instance, 0.1 g. of washed zymine in 6 cc. of buffer,  $p_H$  7.3, will reduce 1 cc. of a 1 : 5000 solution of methylene blue in 1 minute at room temperature. Without lactic acid no reduction occurs. The same is true for yeast. It is able to reduce methylene blue very quickly in the presence of lactic acid. Without the lactic acid the dye is reduced much more slowly. Yet despite this easy and rapid reduction of methylene blue, a vigorous stream of oxygen is necessary before any lactic acid is oxidised in the absence of the dye. This aeration seems a highly artificial condition which does not occur normally in the life of the yeast. The conclusion is that this dehydrase is unable to utilise oxygen and has very little to do with the oxidation of lactic acid unless methylene blue or some other hydrogen acceptor is present. The lactic acid must be oxidised by a system present in smaller concentration or less available so that vigorous aeration is necessary.

The inability of the dehydrase to utilise oxygen may be due to a lack of coenzyme to activate the oxygen. The existence of such a coenzyme seems indicated in muscle. In the presence of lactic acid washed muscle will reduce methylene blue. Szent-Györgyi [1925] has investigated the oxygen uptake of muscle in the presence of lactic acid. By extracting rat's muscle with ice-cold water he finds that the residue is able to reduce methylene blue with lactic acid, but unlike the unextracted muscle, it is unable to take up oxygen. This power is restored if the extract is added to the residue. Thus the extract contains some coenzyme which is able to activate oxygen so that it can be utilised by the lactic acid enzyme. The coenzyme is obviously unnecessary for the methylene blue which does not need activation.

In the following the lactic acid dehydrase has been separated from Delft baker's yeast. In the presence of methylene blue and lactic acid pyruvic acid is formed. This system will take up oxygen because the methylene blue is reduced to methylene white which then takes up oxygen on being reoxidised. No oxygen is taken up or pyruvic acid formed if methylene blue is absent. This fact confirms the inference that the enzyme in yeast is unable to utilise oxygen.

#### *Preparation of the enzyme.*

One pound of baker's yeast is broken up into small pieces and allowed to remain 8 to 10 hours exposed to the air. This allows water to evaporate and makes the treatment with acetone easier and more effective. It is then treated four times with 250 cc. of acetone, each portion being sucked off on a filter pump before the addition of the next. After the last treatment the zymin is dried as quickly as possible either with filter papers or in a vacuum. The resulting fine white powder will keep for months. It is important, however, that the zymin should be prepared quickly for a brown lumpy product does not yield a very active enzyme on extraction. 30 g. of the zymin are then ground up with 100 cc. of *M*/15 disodium hydrogen phosphate and allowed to stand from 3½ to 4 hours at room temperature with intermittent grinding. The zymin is then centrifuged off and the solution which is almost clear is dialysed against distilled water for 6 or 7 hours until the solution in the bag gives a negative nitroprusside test for SH compounds. 3 cc. of the enzyme solution are then used for each experiment. The solution will keep in the ice chest for 24 hours but loses its activity rather quickly after that length of time.

If the extraction is continued much longer than 4 hours or if a stronger phosphate solution is used the succinic acid dehydrase which is present in yeast comes into solution with the lactic acid enzyme. The slightly cloudy solution gives only faint protein tests and only a slight precipitation when 2 % sulphosalicylic acid is added. The faint turbidity is not affected by filtering and the activity of the enzyme remains unchanged. Treatment with charcoal or kaolin resulting in a perfectly clear solution entirely inactivates the enzyme.

The cloudiness is increased when the solution is dialysed and disappears to a great extent when 2 cc. of buffer,  $p_H$  7.3, are added to 3 cc. of the dialysed enzyme. The enzyme however is precipitated after standing for a short time at 37° with a solution of methylene blue. This fact may influence the rate of reduction of the dye to a small extent.

*Specificity and properties of the enzyme.*

The solution of the enzyme is specific for lactic and  $\alpha$ -hydroxybutyric acids. None of the other possible hydrogen donors was activated. These included  $\beta$ -hydroxybutyric, malic, maleic, formic, citric, glutamic, succinic, fumaric, oxalic, acetic, tartaric, and pyruvic acids, and acetaldehyde and glucose. Glyceric acid which was also tried will be discussed later. Oxalic acid, however, has a marked inhibitory effect on the rate of reduction of methylene blue by the enzyme lactic acid system, as little as 0.1 cc. of a 5 % solution in 7 cc. causing an almost complete inhibition. That this is not a property of oxalic acid in general is shown by its entirely negative effect on the citric acid enzyme. Its molecule has in the juxtaposition of two CO groups a structure similar in an important respect to lactic acid, which may enable it to become adsorbed on the enzyme surface and thus keep the lactic acid from being activated.

The importance of this juxtaposition of the CO groups is shown by the fact that  $\alpha$ -hydroxybutyric acid is oxidised whereas  $\beta$ -hydroxybutyric acid is not. The oxidation of  $\alpha$ -hydroxybutyric acid is not quite as rapid as that of lactic acid. A solution of the enzyme which will reduce 1 cc. of a 1 : 5000 solution of methylene blue in the presence of 1 cc. of normal lactic acid in 5½ minutes will reduce the same amount of methylene blue with 1 cc. of normal  $\alpha$ -hydroxybutyric acid in 8 minutes. The presence of an extra carbon atom in the  $\alpha$ -hydroxybutyric acid almost doubles the reduction time of methylene blue.  $\beta$ -hydroxybutyric acid when added to  $\alpha$ -hydroxybutyric acid or to lactic acid does not increase the reduction time of the dye by the last two substances, showing that it is not adsorbed on the enzyme surface.

That pyruvic acid is the product of the action of the enzyme on lactic acid in the presence of methylene blue can be shown by adding to the reduced system a small amount of kaolin, shaking and filtering. The kaolin adsorbs the methylene blue and the clear colourless filtrate then gives the nitroprusside test for pyruvic acid. Pyruvic acid is not further oxidised by the enzyme in the presence of methylene blue so that additions of it to the lactic acid enzyme system ought to cause an inhibition. This is actually found to be the case (Fig. 3).

The inhibition, however, is not due entirely to the presence in excess of the product of the reaction and the consequent slowing of the velocity at which equilibrium is reached, because the pyruvic acid also inhibits the rate at which  $\alpha$ -hydroxybutyric acid is oxidised. In this case it is not the product of the reaction. The most probable explanation is that pyruvic acid is adsorbed

on to the enzyme surface, and not being activated keeps the lactic and  $\alpha$ -hydroxybutyric acids from being oxidised. This might be expected from the fact that oxalic acid is such a powerful poison and pyruvic acid resembles it in the juxtaposition of two CO groups.

Another example of this is glyceric acid. It is not oxidised by the lactic acid enzyme but inhibits the oxidation of lactic acid. Washed zymin is able to oxidise glyceric acid readily, and occasionally preparations of the separated enzyme will oxidise it slowly, indicating that there is a specific enzyme in yeast that will oxidise glyceric acid a part of which may be extracted with the lactic acid enzyme. The difference between the two enzymes is definitely proved by the fact that toluene does not affect the oxidation of the lactic acid at all, whereas the oxidation of the glyceric acid is markedly inhibited. The conclusion is that glyceric acid is adsorbed to a certain extent on the lactic acid enzyme but is not activated, thus causing an inhibition, and this is confirmed by the fact that enzyme extracts, which will not oxidise glyceric acid at all, are still inhibited by glyceric acid.

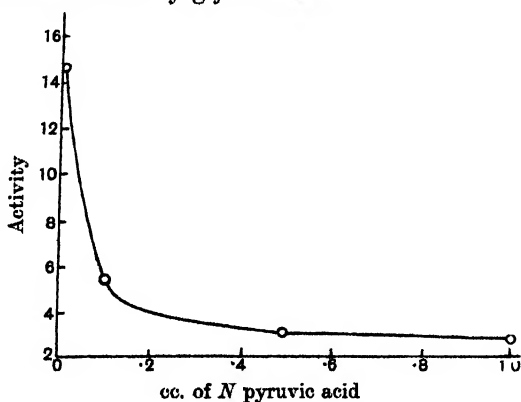


Fig. 3. Effect of pyruvic acid on the reduction time of methylene blue by lactic acid.

These results confirm those of Quastel and Wooldridge [1928] on the effect of various substances containing CO groups in juxtaposition on the oxidation of lactic acid by *B. coli*. Thus the idea that a molecule containing two contiguous CO groups is adsorbed on the enzyme and either inhibits the oxidation of the lactic acid or is oxidised itself seems valid. Exp. 1 shows the effect of glyceric acid on the oxidation of lactic acid, and Exp. 2 shows the effect of pyruvic acid on the oxidation of  $\alpha$ -hydroxybutyric acid. Exp. 3 shows the effect of toluene on the oxidation of lactic and glyceric acids.

*Exp. 1.* 3 cc. of the isolated enzyme, 2 cc. of buffer,  $p_H$  7.3, and 1 cc. of 1 : 5000 methylene blue were placed in each of three tubes. Tube A also contained 1 cc.  $N/2$  lactic acid and 1 cc. water, tube B 1 cc.  $N/2$  lactic acid and 1 cc.  $N/2$  glyceric acid, and tube C 1 cc.  $N/2$  glyceric acid and 1 cc. water. The acids were added as sodium salts, and the experiment was carried out *in vacuo* at 37°.

Tube	Reduction time in minutes
A	8
B	17
C	40

*Exp. 2.* 3 cc. of separated enzyme, 2 cc. of buffer,  $p_H$  7.3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc.  $N/2$  lactic acid and 1 cc. water, tube B 1 cc.  $N/2$  lactic acid and 1 cc.  $N/2$  pyruvic acid, tube C 1 cc.  $N/2$   $\alpha$ -hydroxybutyric acid and 1 cc. of water, and tube D 1 cc.  $N/2$   $\alpha$ -hydroxybutyric acid and 1 cc.  $N/2$  pyruvic acid. The conditions were the same as in Exp. 1.

Tube	Reduction time in minutes
A	12
B	20
C	18
D	30

*Exp. 3.* 3 cc. of separated enzyme, 2 cc. of buffer,  $p_H$  7.3, and 1 cc. of 1 : 5000 methylene blue were placed in each of four tubes. Tube A also contained 1 cc.  $N/2$  lactic acid, tube B the same plus 0.1 cc. toluene, tube C 1 cc.  $N/2$  glyceric acid instead of the lactic acid, and tube D the same plus 0.1 cc. toluene. The conditions were the same as in the other experiments.

Tube	Reduction time in minutes
A	9
B	8
C	40
D	90

The rate of reduction of methylene blue was measured when varying quantities of lactic acid were present. The resulting curve (Fig. 4) differs markedly from that obtained for the citric acid enzyme in that increasing quantities of the substrate do not cause an inhibition. This is true both for lactic acid and for  $\alpha$ -hydroxybutyric acid (Fig. 5). It might be possible to differentiate the dehydrases on this basis: on the one hand, the citric acid and xanthine enzymes which are inhibited by an excess of substrate, and on the other the lactic and succinic enzymes which are not. The curves for lactic and  $\alpha$ -hydroxybutyric acids are similar. The velocity of reduction falls off in both cases when just under 0.5 cc.  $N$  acid is present. The figures are given in Figs. 4 and 5 as cc. in 7 cc. Consequently the velocity falls off when the concentration becomes less than  $N/14$ , a concentration which is far above the theoretical necessary to reduce the methylene blue present. In this respect too the difference from the citric acid enzyme should be noted, for the maximum concentration of substrate for the latter is about  $N/200$ .

Besides methylene blue oxygen was tried as a hydrogen acceptor. No uptake could be detected when the enzyme was shaken in a Barcroft apparatus in the presence of lactic acid. If methylene blue were added to the solution an oxygen uptake occurred because the dye was reduced by the enzyme-lactic



acid system and was reoxidised again by the oxygen present. In this case pyruvic acid can be shown to be present whereas no test can be obtained if the enzyme and lactic acid are shaken in oxygen without methylene blue. The enzyme suffers a certain loss of activity after being shaken in air for a length of time. As there is plenty of catalase present in the solution this loss cannot be due to a destruction by hydrogen peroxide. It is probably caused by a partial coagulation of the colloidal particles in the solution to form larger particles with less enzyme surface. An increasing turbidity is observed after shaking in air, suggesting that this may be the case.

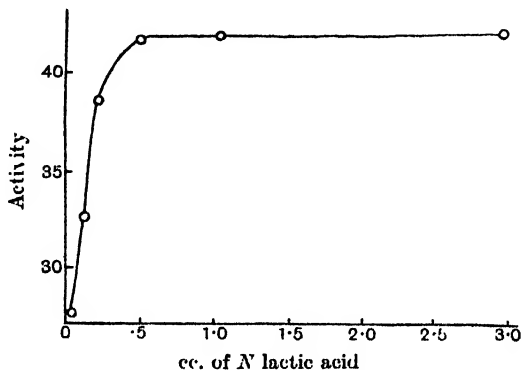


Fig. 4. Effect of varying concentrations of lactic acid on the reduction of methylene blue.

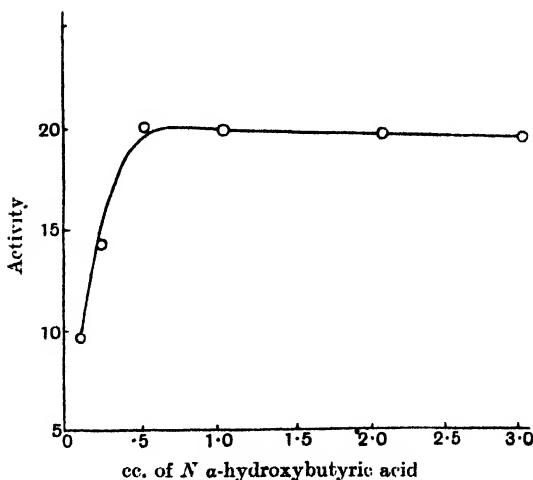


Fig. 5. Effect of varying amounts of  $\alpha$ -hydroxybutyric acid on the reduction of methylene blue.

An attempt was made to supply active oxygen to the enzyme-lactic acid system on the possibility that pyruvic acid might be formed without the intervention of methylene blue. In order to do this a solution of xanthine oxidase, hypoxanthine, and a strong solution of peroxidase were added to the lactic acid enzyme and lactic acid. Oxygen was then bubbled through the

solution for several hours at 37°. The solution was then tested for pyruvic acid but with negative results. The peroxidase solution was made strong so that it could successfully compete with the catalase for the hydrogen peroxide.

Inorganic nitrate is not reduced. *m*-Dinitrobenzene gives a yellow colour when placed in a vacuum tube with the enzyme-lactic acid system, but this does not change to a purple on the addition of soda. If the reduction is allowed to go on for a long time, 5 or 6 hours, with a strong enzyme solution which will reduce 1 cc. of 1 : 5000 methylene blue in 10 minutes, a faint purple colour is seen on the addition of soda. It is obvious from the yellow colour produced that a reduction does take place but it seems to differ from the ordinary one found by Lipschitz for the dehydrases of tissue, the lactic acid one included. The possible explanation is that another reduction product is formed along with traces of the usual nitrophenylhydroxylamine. These traces would account for the faint purple colour with soda, and the other reduction product for the deep yellow.

#### DISCUSSION.

The work done on the reduction of methylene blue by tissues and bacteria is summarised in Table I. Frog's muscle (Thunberg) and *B. coli* [Quastel and Whetham, 1925; Quastel and Wooldridge, 1925] have been most extensively studied in regard to their ability to reduce methylene blue in the presence of various substances. Quastel and Wooldridge [1927] have treated *B. coli* in various ways showing that specific activations are destroyed by various treatments. These observations are very extensive and are not included in the table. The specificity of the alkaline phosphate extracts of washed muscle and liver is taken chiefly from Wishart. He found that a few substances such as formic acid and ketoglutaric acid were activated to a small extent by the extract, but the rates of reduction were so small compared to the rate with succinic acid that they are put as negative in the table. The data for the milk are taken from Dixon [1926], for the potato from Bernheim, and for dried yeast from Harden and Norris. Rat's muscle has been studied but not so completely (Bernheim and Dixon). The list of substances in the following table includes most of those that are active with either *B. coli* or frog's muscle. A + sign indicates that the substance will reduce methylene blue rapidly in the presence of the tissue or extract, a - sign that it will not reduce at a detectable rate. A blank space means that the substance still remains to be tested. Fumarase which is not a dehydrase has been included in the table.

From a consideration of the table there are three types of evidence for the specificity of the dehydrases. The first is the separation of the enzyme from the tissue. This evidence is fairly conclusive for the separation depends on differences in physical properties of the enzymes, *i.e.* the centres responsible for the activations are attached to different colloids. It would be difficult to explain these facts on the assumption of one enzyme in the tissue which was originally capable of effecting all the activations but which during the process of

Table I.

Tissue or extract	Acetaldehyde	Other aldehydes	Hypoxanthine	Alanine	Tryptophan	Histidine	Formic acid	Normal fatty acids to capronic*	iso-Valeric acid	Citric acid	Glutamic acid	Glyceric acid	Hydroxyglutaric acid	Lactic acid	$\alpha$ -Hydroxybutyric acid	$\beta$ -Hydroxybutyric acid	Maleic acid	Malic acid	Succinic acid	Fumaric to malic acid	Tartaric acid	Glycollic acid	Certain hexoses and pentoses	Certain alcohols	Certain disaccharides
Washed frog's muscle ... ..	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Frog's muscle treated with liquid air	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Washed rat's muscle ... ..	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
M/14 Na <sub>2</sub> HPO <sub>4</sub> extract of washed ox's muscle	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Same heated $\frac{1}{2}$ hour at 50°	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
M/14 Na <sub>2</sub> HPO <sub>4</sub> extract of washed ox's liver	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Water extract of acetone liver	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Resting <i>B. coli</i> ... ..	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Water extract of autolysed <i>B. coli</i>	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Dried yeast (Lebedeff)	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
M/14 Na <sub>2</sub> HPO <sub>4</sub> extract of acetone yeast	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Milk ... ..	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Potato ... ..	...	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

\* Dixon (unpublished) has found that propionic acid is oxidised by rat's muscle and Thunberg (privately communicated) has found the same for frog's muscle. In his original paper Thunberg [1920] states that it is not oxidised.

extraction has been so altered as to appear specific for one substrate. Four dehydrases have been separated in this way: the succinic, lactic, citric and xanthine.

The second type of evidence is suggestive but not conclusive. It consists of treating the tissue in various ways, destroying certain activations and leaving others. Thus frog's muscle after treatment with liquid air will only activate succinic and hydroxyglutaric acids. The succinic acid dehydrase has by extraction been obtained free from the hydroxyglutaric acid enzyme, therefore it is justifiable to assume that the oxidation of the latter acid is due to a distinct enzyme. Then the water extract of autolysed bacteria will only oxidise lactic, glyceric and  $\alpha$ -hydroxybutyric acids. As the original *B. coli* will oxidise  $\beta$ -hydroxybutyric acid this proves that there are two different enzymes responsible for the oxidation of  $\alpha$ - and  $\beta$ -hydroxybutyric acids. Similarly zymine will oxidise lactic, glyceric and  $\alpha$ -hydroxybutyric acids, but extracts can be obtained that will only oxidise lactic and  $\alpha$ -hydroxybutyric acids, indicating that the oxidation of glyceric acid is due to a distinct enzyme. These arguments are not conclusive, however, until extracts are obtained which will oxidise  $\beta$ -hydroxybutyric or glyceric acid and not lactic and  $\alpha$ -hydroxybutyric acids. Another type of argument is also possible. The enzyme from milk will oxidise purines and aldehydes, that from potato only aldehydes, and therefore the milk enzyme may be really composed of two specific entities. But as the xanthine oxidase from milk has been concentrated 4000 times and still retains the ability to oxidise aldehydes [Dixon and Kodama, 1926] it would be unsafe to assume this until further evidence is available. From these facts, then, the existence of three further specific dehydrases is indicated, namely, the enzymes that will oxidise hydroxyglutaric,  $\beta$ -hydroxybutyric and glyceric acids.

#### SUMMARY.

1. Methods for the separation of the citric acid dehydrase from liver and the lactic acid dehydrase from yeast have been described.
2. The specificity and properties of these two enzymes have been studied.
3. The general question of the specificity of the dehydrases has been discussed.

My thanks are due to Sir F. G. Hopkins for his interest and encouragement, to Dr Malcolm Dixon for his advice and help, and to Dr J. H. Quastel for suggesting certain experiments.

## REFERENCES.

- Alwall (1928). *Skand. Arch. Physiol.* **54**, 1.  
 Amberg and McClure (1924). *Amer. J. Physiol.* **44**, 452.  
 Battelli and Stern (1911). *Biochem. Z.* **31**, 478.  
 Bernheim (1928). *Biochem. J.* **22**, 344.  
 Bernheim and Dixon (1928). *Biochem. J.* **22**, 125.  
 Dakin (1921). *Physiol. Rev.* **1**, 394.  
 Dixon (1926). *Biochem. J.* **20**, 703.  
 Dixon and Kodama (1926). *Biochem. J.* **20**, 1104.  
 Dixon and Thurlow (1924). *Biochem. J.* **18**, 976.  
 Fürth and Lieben (1922, 1). *Biochem. Z.* **128**, 144.  
 ——— (1922, 2). *Biochem. Z.* **132**, 165.  
 Greenwald (1915). *J. Biol. Chem.* **18**, 115.  
 Harden and Norris (1915). *Biochem. J.* **9**, 330.  
 Hoffert (1926). *Biochem. J.* **20**, 358.  
 Lipschitz (1921). *Pflüger's Arch.* **191**, 1.  
 Meyerhof (1919). *Pflüger's Arch.* **175**, 20.  
 Morgan (1926). *Biochem. J.* **20**, 1281.  
 Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.  
 Quastel and Whetham (1925). *Biochem. J.* **19**, 520, 645.  
 Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.  
 ——— (1927). *Biochem. J.* **21**, 148, 1224.  
 ——— (1928). *Biochem. J.* **22**, 689.  
 Salant and Wise (1907). *J. Pharm.* **11**, 123.  
 Stephenson (1928). *Biochem. J.* **22**, 605.  
 Szent-Gyorgyi (1925). *Biochem. Z.* **157**, 50.  
 Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.  
 Wishart (1923). *Biochem. J.* **17**, 103.

## CXLVIII. SUCCINOXIDASE.

### II. INFLUENCE OF PHOSPHATE AND OTHER FACTORS ON THE ACTION OF THE SUCCINODEHYDROGENASE AND THE FUMARASE OF LIVER AND MUSCLE.

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*(Received July 18th, 1928.)*

MUSCLE, liver and other tissues have been shown [Thunberg, 1909; Batelli and Stern, 1911] to contain a very powerful and remarkably specific enzyme, succinoxidase, which, according to Batelli and Stern, converts succinic into optically inactive malic acid. Einbeck [1914] regarded fumaric and not malic acid as the sole product, but later [1919] showed that the enzyme first converts succinic to fumaric acid and then, by a balanced reaction, to a mixture of fumaric (25 %) and malic (75 %) acids. Dakin [1922] showed that fumaric acid, reacting with muscle pulp, formed exclusively *laevo*-malic and not the inactive acid.

A number of attempts have been made to isolate the enzyme. Thus Batelli and Stern extracted it by means of dilute sodium hydroxide but obtained an enzyme of very low activity. Widmark [1918] improved the method by using 1.5 % sodium carbonate. Ohlsson [1921] substituted a  $M/15$  solution of disodium hydrogen phosphate and this method has been generally used. Modifications, largely of the method of washing the muscle previous to extraction, have been introduced by Grönwall [1923], Svensson [1923], Sahlin [1924] and finally by Anderson [1927], who extracted the muscle several times with dilute saline before using  $M/15$  phosphate, thus obtaining an enzyme extract considerably freer from proteins. All these investigators, however, have followed the course of the action of the enzyme in terms of the reduction times of methylene blue, using the usual Thunberg technique. Recently, Hahn and Haarmann [1927, 1928] and Fischer [1927] have shown that washed muscle, with sufficient methylene blue and sodium succinate, gives rise anaerobically to the same products, fumaric and malic acids, which it produces aerobically, and it seems likely therefore, that the enzyme system is the same in the two types of experiments.

In a recent paper [Clutterbuck, 1927] a polarimetric method was described for the determination of succinic acid in muscle and liver pulp in terms of its conversion into *l*-malic acid. Using this method, it was shown that liver was

eight times as active as the same moist weight of muscle in converting succinic or fumaric acid into malic acid, and cyanide, although completely inhibiting the oxidation or dehydrogenation of succinic acid, had no effect on the conversion of fumaric to malic acid. These experiments emphasised the fact that at least two enzymes were present in the oxidase system, a true succinodehydrogenase converting succinic to fumaric acid and a fumarase, converting fumaric to malic acid. This polarimetric method permits the study of both reactions and of the effect of various factors, other than cyanide, upon them. Also, by its means some preliminary experiments have been carried out, which are designed to discover the best method of separating the two enzymes from tissue and from one another.

#### SOLUBILITY AND STABILITY OF SUCCINODEHYDROGENASE AND FUMARASE.

One of the most striking differences, apart from the action of cyanide, between the fumarase and dehydrogenase is seen in respect to their solubilities and stabilities. The opalescent enzyme solution obtained by steeping washed rabbit's muscle in phosphate solution and centrifuging readily reduces methylene blue in presence of succinate and absence of oxygen and therefore contains the dehydrogenase. If, however, the solution is shaken a little by hand, the enzyme is completely inactivated. Moreover, when the phosphate enzyme solution or the aqueous extract of muscle is placed in a bottle with sodium succinate solution and rotated, the air being displaced by oxygen as in the experiments previously described [Clutterbuck, 1927], it was found that even the gentle rotational movement necessary to ensure oxygenation, although accompanied by only slight frothing, was sufficient to prevent any oxidation of succinate. During the rotation, however, a clot of coagulated protein formed, but the opalescence only slightly decreased. The clot probably contained the inactivated dehydrogenase, whilst the remaining turbidity was due to the fact that the fumarase protein complex under these conditions is quite stable, and coagulates only to a slight extent. A phosphate enzyme extract, rotated thus with sodium fumarate solution, showed in fact a six times greater fumarase activity than the original muscle pulp. Succinoxidase has also always been regarded as an extremely insoluble enzyme, only elutable by alkaline (phosphate, carbonate, etc.) solutions and after elution being readily precipitable by bringing the  $p_H$  of the solution to 6.5. It has been found, however, that although the dehydrogenase could not be detected in the aqueous extracts by our method, owing most probably to its inactivation during rotation, yet active fumarase is readily washed out of muscle by water or dilute (0.25 %) saline and is not precipitated to any great extent at  $p_H$  6.5.

#### *The "active" and "elutable" fumarase of muscle and liver pulp.*

A number of experiments were therefore carried out in which the minced liver or muscle pulp (of rabbit) was steeped in an equal weight of water or 0.25 % saline, placed in a fine cloth and the fluid expressed. This process was

repeated 16 times and the extracts combined in fours and the activity determined polarimetrically. In order conveniently to compare the activity of the extracts, the production of 1.22 g. *l*-malic acid in 1 hour at 38° using 500 cc. of 1 % fumaric acid, as sodium salt, is called unit activity. The following results were obtained:

Muscle (150 g.)		Liver (50 g.)	
Saline or aqueous extracts	Fumarase activity units	Saline or aqueous extracts	Fumarase activity units
1-4	1.0	1-4	4.0
4-8	0.5	4-8	2.0
8-12	0.25	8-12	0.5
12-16	0.20	12-16	0.3

It will be seen that the amount of free or "active" enzyme, removed in the aqueous extracts of muscle and liver pulp, has approximately the same activity as the original pulp.

The muscle and liver residues were then steeped, on the one hand, in an equal weight of *M*/15 phosphate for an hour and, on the other, in an equal weight of *M*/15 bicarbonate solution to which a little carbonate had been added to bring the  $p_H$  approximately to that of the phosphate, namely about 9, and the extracts were then expressed as before. This process was repeated three times and the activity of each extract was determined. Finally the tissue residue was steeped twice for 24 hours and the activity of the extract found. Using phosphate as the eluting agent, expressing the results in units as above, we obtain the following results:

Muscle (150 g.)		Liver (50 g.)	
Phosphate extracts	Fumarase activity units	Phosphate extracts	Fumarase activity units
1 after 1 hour	4.0	1 after 1 hour	0.25
2 " 1 "	2.0	2 " 1 "	0.25
3 " 1 "	0.6	3 " 1 "	0.25
4 " 24 hours	0.6	4 " 24 hours	1.0
5 " 24 "	0.5	5 " 24 "	0.8

Using bicarbonate as eluting agent the results were similar but the activity was always somewhat smaller.

If we now convert the figures of our previous paper into units, whereas the fumarase activity of 100 g. muscle pulp was 1 unit, that of 100 g. liver pulp was about 8 units. Comparing these figures with those of the two sets of figures above, we obtain the following results.

(1) Liver pulp, weight for weight, has 8 or 10 times as great a fumarase activity as muscle pulp, but this only indicates that the liver pulp contains 8 or 10 times as much free or "active" enzyme as muscle.

(2) An amount of fumarase is removed from muscle and from liver by steeping 8 times in an equal weight of water or saline, approximately equal in activity in each case to the activity of the tissue pulp itself.

(3) After the amount of enzyme, extracted by this treatment with water or saline, has become reduced to a very small amount, steeping in *M*/15



phosphate or in a bicarbonate-carbonate buffer of the same  $p_H$  and expressing liberates from the muscle residue a further large amount of enzyme, having in the several extracts six times the activity of the original pulp, whereas similar treatment of the liver residue, the volume of which is however much smaller than the muscle residue, removes only a small amount of enzyme.

(4) The total activity of the water and phosphate extracts of 50 g. liver was approximately equal to the total activity of the extracts of 150 g. muscle.

It is concluded, therefore, that, although in the pulp liver appears 8 or 10 times as active as muscle in converting fumaric to malic acid, yet the total amount of enzyme, free and elutable, of liver is only about three times as much as in muscle, and, whereas almost the whole of the liver fumarase is free and active in the pulp emulsion, only about one-seventh of the total muscle fumarase is free and active, the remainder being readily eluted by alkaline bicarbonate-carbonate or phosphate buffer solution of  $p_H$  about 9. Borate buffer at this  $p_H$  gelatinised the muscle and no extract could be expressed. Washing the liver or muscle pulp with water or 0.25 % saline removes only the free, active enzyme, *i.e.* removes one-seventh of the total muscle enzyme but almost all of the liver enzyme. Previous workers, using washed muscle, have always employed either pulp in phosphate buffer or a phosphate enzyme extract of muscle and they have, therefore, by their technique, eluted and used in an activated condition that part of the enzyme not removed by washing.

*The influence of phosphate on the action of the fumarase of muscle pulp.*

That the activity of muscle fumarase is greater in phosphate than in bicarbonate buffer at the same  $p_H$  (7.2) is seen from the following experiment in which a sample of muscle (50 g.) was rotated, on the one hand, with fumarate (500 cc. 1 %) as control and, on the other, with fumarate to which had been added phosphate and bicarbonate sufficient to make the concentration 0.05 *M*, the  $p_H$  in all three cases being adjusted to 7.2. The following figures were obtained polarimetrically for the amount of *l*-malic acid produced.

Time (hours)	Control. <i>l</i> -Malic g.	+ Phosphate (0.05 <i>M</i> ). <i>l</i> -Malic g.	+ Bicarbonate (0.05 <i>M</i> ). <i>l</i> -Malic g.
0.5	0.45	1.30	0.60
1.0	0.78	2.22	1.05
1.5	1.26	2.66	1.61
2.0	1.55	2.90	1.97

It would appear therefore that phosphate, in addition to having the power (like bicarbonate) to elute the enzyme in virtue of its alkalinity, has also a direct activating effect on the fumarase. This activating effect was next investigated.

A large number of experiments with muscle pulp and fumarate have now been carried out and it has been found that the initial fumarase activity of the pulp varies considerably from animal to animal and often appears to be

related to the condition of the animal. In all the experiments recorded in this paper the usual apparatus was therefore duplicated, two bottles being rotated at the same speed in the same thermostat, one containing muscle and fumarate as control and the other muscle, fumarate and phosphate or other added substance. The usual 25 cc. sample was taken simultaneously from the two bottles, coagulated by heat and uranium acetate added. It was found that, if the flasks were then allowed to stand for half an hour, 75 cc. water being then added and allowed to stand for a further short period, the occasional difficulty previously reported of obtaining a clear filtrate for polarimetric observation never arose.

When phosphate is added to muscle and liver pulp and rotated with sodium fumarate solution, the rate of conversion to malate is greatly accelerated. That this is not a  $p_H$  effect, may be proved by adjusting the  $p_H$  of the two bottles to say 7.2 before the experiment when the same phosphate activation is still observed. Moreover, if a sample of the same pulp is caused to react with fumarate on the one hand in phosphate buffer, and on the other in borate buffer solution of the same  $p_H$ , whereas in the latter the rate is practically normal (there is actually a slight inhibition), in the phosphate buffer the usual acceleration is obtained. It would appear that the  $p_H$  optimum for fumarase covers a fairly wide range from about 6 to 9, and that changes of  $p_H$  over this range have relatively a slight effect on the rate of the reaction. The following results are typical examples obtained in a number of experiments using 50 g. minced sterile muscle pulp with 500 cc. 1 % fumaric acid (as sodium salt) rotating at 38° and comparing with a second similar sample of the same muscle and fumarate containing various amounts of added phosphate, the  $p_H$  in all cases being adjusted to 7.2. The figures give the *l*-malic acid (g.) produced, as determined polarimetrically.

Time (hours)	0.25	0.50	0.75	1.00	1.50	2.00
	<i>l</i> -Malic produced (g.)					
Control	0.31	0.67	0.97	1.32	1.82	—
+ 0.013 <i>M</i> phosphate	0.43	0.91	1.26	1.52	2.12	—
Increase of <i>l</i> -malic (g.)	0.12	0.24	0.29	0.30	0.30	—
Control	0.50	0.81	1.11	1.46	2.02	—
+ 0.026 <i>M</i> phosphate	0.63	1.14	1.65	2.02	2.64	—
Increase of <i>l</i> -malic (g.)	0.13	0.33	0.54	0.56	0.62	—
Control	0.34	0.61	0.90	1.14	1.55	2.02
+ 0.052 <i>M</i> phosphate	0.78	1.35	1.87	2.28	2.69	2.94
Increase of <i>l</i> -malic (g.)	0.44	0.74	0.97	1.14	1.14	0.92
Control	0.41	0.84	1.16	1.55	2.15	2.62
+ 0.078 <i>M</i> phosphate	0.47	1.03	1.41	2.11	2.82	3.22
Increase of <i>l</i> -malic (g.)	0.06	0.19	0.25	0.56	0.67	0.60
Control	0.40	0.80	1.15	1.50	2.10	—
+ 0.104 <i>M</i> phosphate	0.48	1.00	1.58	2.06	2.67	—
Increase of <i>l</i> -malic (g.)	0.08	0.20	0.43	0.56	0.57	—
Control	0.55	0.93	1.30	1.69	2.34	—
+ 0.156 <i>M</i> phosphate	0.61	1.22	2.00	2.62	3.29	—
Increase of <i>l</i> -malic (g.)	0.06	0.29	0.70	0.93	0.95	—

If the results of two of these experiments are graphed (Fig. 1), namely those with 0.052 *M* phosphate (which shows optimal activity) and with 0.156 *M* phosphate, it is seen that with the former, as also in experiments with smaller amounts of phosphate, the curve with phosphate (*A*) is regular and lies above the control (*B*), whereas with larger than optimal amounts (*C, D*)

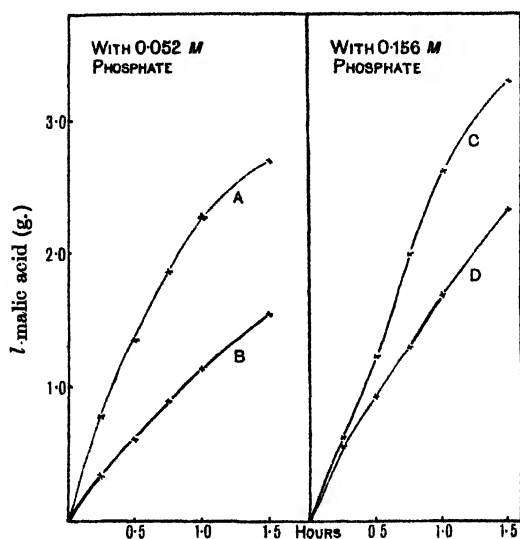


Fig. 1.

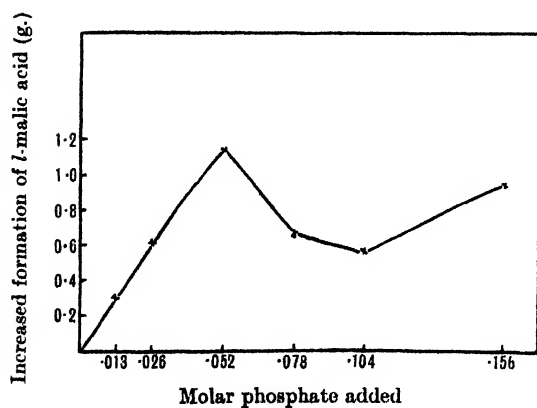


Fig. 2.

there appears at first an inhibition which is only overcome after about half an hour. The control curves for the muscle (*B, D*) of different amounts are all similar but vary considerably in steepness, and the activity in those muscles which have been examined in spring and summer appears on the whole to be higher than that of the muscle of animals previously recorded, which were examined in autumn and winter.

The effect of the phosphate concentration is best seen by plotting the concentration of added phosphate against the increased amount of *l*-malic acid found after 1.5 hours (Fig. 2).

From this curve it is seen that the increase in the formation of *l*-malic acid is almost directly proportional to the increase in phosphate concentration until the latter has increased to about 0.05 *M*. This appears to be an optimal concentration and with further increase the curve at first falls and then rises again. It is interesting to note that this optimum is identical with the phosphate optimum obtained by Platt and Dawson [1926] for the action of pancreatic lipase.

*Effect of phosphate on the rate of dehydrogenation by muscle pulp.*

Since the action of succinodehydrogenase is so very much slower than that of fumarase, it should be possible by carrying out similar experiments, with and without phosphate, replacing fumaric with succinic acid, to detect any effect of phosphate on the rate of dehydrogenation. Rotating 50 g. muscle in 500 cc. of 1 % succinic acid solution (as sodium salt) at 38° in an atmosphere of oxygen as control and a similar suspension containing phosphate (0.05 *M*), no influence on the rate of the dehydrogenation could be detected.

*Influence of phosphate on the activity of liver pulp and of aqueous extracts of muscle and liver pulp.*

The fumarase of liver pulp is activated by phosphate in the same way as that of muscle pulp. Thus, with one of the most active samples of liver that we have examined, the difference in the activity with and without the optimal amount of added phosphate was of the same order as in the muscle experiments; *e.g.* rotating 15 g. liver with 500 cc. of 1 % fumarate at 38° as control and a similar sample to which phosphate was added to make the concentration 0.05 *M*, the  $p_{\text{H}}$  being adjusted in both cases to 7.2, the following results were obtained.

Time (hours)	Control		+ 0.05 <i>M</i> phosphate		Increase of <i>l</i> -malic (g.)
	Rotation	<i>l</i> -Malic (g.)	Rotation	<i>l</i> -Malic (g.)	
0	0	0	0	0	0
0.25	1.02	1.14	1.44	1.61	0.47
0.50	1.74	1.95	2.44	2.73	0.78
0.75	2.10	2.35	3.07	3.44	1.09

Phosphate not only activates the fumarase of the tissue pulps but also activates the free eluted enzyme of the aqueous extracts of the pulps. Thus with 30 g. liver, eight aqueous extracts were combined, made up to 400 cc. and divided into two equal portions. One sample was rotated with fumarate, the final concentration being 1 % as before and the other with fumarate and sufficient phosphate to make the concentration optimal, the final  $p_{\text{H}}$  in both cases being 7.2. The results were as follows.

Time (hours)	Control		+ 0.05 M phosphate		Increase of <i>l</i> -malic (g.)
	Rotation	<i>l</i> -Malic (g.)	Rotation	<i>l</i> -Malic (g.)	
0	0	—	—	—	0
0.25	0.72	0.81	1.41	1.58	0.77
0.50	1.25	1.40	2.32	2.60	1.20
0.75	1.65	1.85	2.79	3.12	1.27
1.00	1.98	2.22	3.06	3.42	1.20

Similarly with the enzyme of aqueous extracts of muscle pulp, phosphate activates almost to the same extent as in the pulp experiments.

*Influence of temperature on the fumarase activity of liver and muscle pulp.*

The fumarase of liver, as we have already shown, is almost entirely in the free active condition and we might expect, therefore, to find that the activity of the enzyme, on rotating with fumarate at different temperatures, would change according to the well-known rule, namely, that for every rise of 10° the rate of reaction would be doubled or trebled. This was found to be the case. Thus with 15 g. of a very active sample of liver the following figures were obtained.

15 g. liver, 500 cc. fumarate, $p_H$ 7.2, temp. 39°		15 g. liver, 500 cc. fumarate, $p_H$ 7.2, temp. 19°	
Time (hours)	<i>l</i> -Malic formed (g.)	Time (hours)	<i>l</i> -Malic formed (g.)
0	0	0	0
0.25	1.00	1	0.96
0.50	1.80	2	1.67
0.75	2.46	3	2.29
1.00	2.84	4	2.83
1.50	3.40	5	3.24
2.00	3.44	6	3.44

The rate at 39° at the beginning of the experiment was almost exactly four times that at 19° but subsequently fell to three times.

With a much less active sample of liver, the activity at 39° was five or six times that at 19° and on plotting the results (see Fig. 3) it is seen that with the less active sample at the lower temperature, there is a tendency for the curve to become more S-shaped. The figures for the less active liver are the following.

Time (hours)	<i>l</i> -Malic formed at $p_H$ 7.2, temp. 39°	Time (hours)	<i>l</i> -Malic formed at $p_H$ 7.2, temp. 19°
0	0	0	0
0.25	0.39	0.5	0.15
0.50	0.90	1.0	0.32
0.75	1.50	1.5	0.52
1.00	2.06	2.0	0.76
1.25	2.50	2.5	1.12
1.50	3.01	3.0	1.58
2.00	3.42	4.0	2.37
2.50	3.46	5.0	2.85
		6.0	3.22
		7.0	3.44

With muscle pulp, however, the influence of temperature appears to be somewhat different. We have already shown that only about one-seventh of the total elutable enzyme of muscle is free and active whilst the rest is adsorbed or in some way held in an inactive form but is elutable by phosphate or

bicarbonate-carbonate buffer of  $p_H$  9.0. Using muscle pulp (50 g.) the activity as is seen from the accompanying figures is only twice as great at  $39^\circ$  as at  $19^\circ$ .

Time (hours)	<i>L</i> -Malic formed at $p_H$ 7.2, temp. $39^\circ$	Time (hours)	<i>L</i> -Malic formed at $p_H$ 7.2, temp. $19^\circ$
0	0	0	0
0.33	0.60	0.33	0.34
0.66	1.15	0.66	0.60
1.00	1.72	1.00	0.82
1.50	2.22	1.50	1.14
2.00	2.79	2.00	1.46
2.50	2.96	2.50	1.72

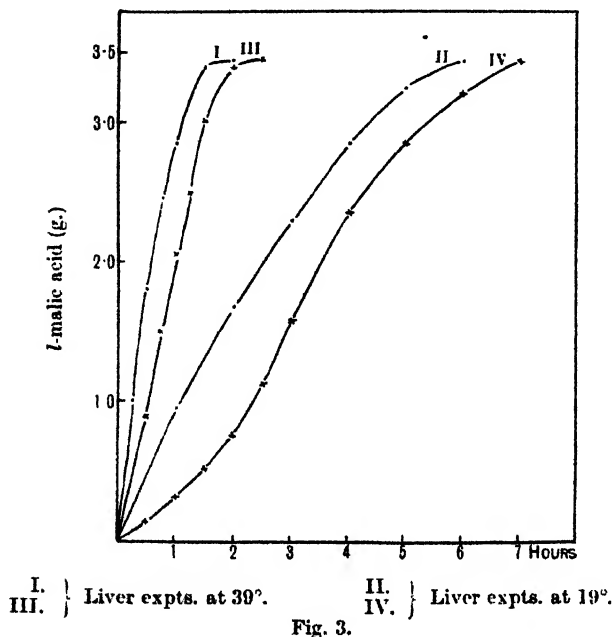


Fig. 3.

It is remarkable that the temperature coefficient with muscle pulp is so much smaller. Whether this is an adsorption effect or due to the production of some inhibitory substance at the higher temperature is not clear, but the former view is somewhat supported by the fact that if in place of the muscle pulp we use the aqueous extract of the muscle, then the coefficient is a little larger but even then it does not double every  $10^\circ$  as in the case of the liver enzyme. Thus, eight aqueous extracts of 100 g. muscle were combined, the  $p_H$  was adjusted to 7.2, the liquid made up to 800 cc. and divided into two equal portions and rotated with fumarate, one at  $39^\circ$  and the other at  $19^\circ$ . The results were as follows.

Time (hours)	<i>L</i> -Malic formed at $39^\circ$ (g.)	<i>L</i> -Malic formed at $19^\circ$ (g.)
0	—	—
0.33	0.47	0.21
0.66	0.86	0.32
1.00	1.28	0.47
1.50	1.50	0.60
2.00	1.79	0.87
2.50	2.22	0.94

At the end of the first hour the rate at 39° was roughly three times that at 19°.

*The influence of other factors on fumarase and succinodehydrogenase activity.*

*Sodium chloride* appears to inhibit very considerably the action of succinodehydrogenase, but only slightly the action of fumarase. Thus rotating 15 g. liver with 500 cc. 1 % succinate solution at 38° as control and a similar sample containing in addition 0.9 % sodium chloride, the following figures were obtained.

Time (hours)	Control. L-Malic (g.)	+ 0.9 % sodium chloride. L-Malic (g.)	Decrease of L-malic (g.)
0	—	—	—
2	0.50	0.22	0.28
4	1.25	0.38	0.87
5	1.60	0.52	1.08

Replacing succinate with fumarate, the inhibition of fumarase by 0.9 % sodium chloride is relatively very small.

Time (hours)	Control. L-Malic (g.)	+ 0.9 % sodium chloride. L-Malic (g.)	Decrease of L-malic (g.)
0	—	—	—
3	3.81	3.36	0.45

*Sodium fluoride*, on the other hand, has a well-marked inhibitory effect on fumarase. Thus rotating 50 g. muscle with 500 cc. 1 % fumarate at 38° as control and comparing with a similar sample containing also 1 % sodium fluoride, the following results were obtained.

Time (hours)	Control L-Malic (g.)	+ 1 % sodium fluoride. L-Malic (g.)	Decrease of L-malic (g.)
0	—	—	—
0.25	0.28	0.10	0.18
0.50	0.50	0.22	0.28
0.75	0.67	0.29	0.38
1.00	0.91	0.46	0.45
1.50	1.25	0.78	0.47
2.00	1.52	1.08	0.44
3.00	2.11	1.64	0.47

*Calcium chloride* (4 %) caused a slight inhibition, and calcium nitrate a much greater inhibition, of fumarase activity.

*Bile salts* (2 %) caused a very slight acceleration.

#### *Presence of fumarase in invertebrate muscle.*

*Pecten maximus*. In his recent investigation on the chemical changes in muscle, Boyland [1928] found that succinic acid was present in extracts of the muscles of several invertebrates, particularly in *Pecten*. It seemed reasonable to expect, therefore, that the enzyme system would also be present. The large adductor muscles of several specimens of *Pecten* were minced, and 100 g. pulp rotated as before with fumarate at 17°. The following results show that fumarase is present in these muscles.

Time (hours)	L-Malic acid (g.)
0	—
1	0.16
2	0.38
3	0.50
4	0.63
5	0.77

*Anodonta cygnea*. The adductor muscles and the muscular part of the foot of a number of *Anodonta* were minced and 110 g. of the pulp rotated with fumarate at 18°. The muscle appeared about twice as active as the sample of pecten muscle examined above.

Time (hours)	L-Malic acid (g.)
0	—
1	0.26
2	0.59
3	0.98
4	1.37
5	1.77

*Presence of fumarase in blood cells.*

Defibrinated fresh ox-blood was centrifuged and the corpuscles and serum rotated with fumarate at 37°. The corpuscles showed a small activity but the serum did not appear to contain fumarase. The results with 80 g. corpuscles were as follows.

Time (hours)	L-Malic acid (g.)
0	—
0.5	0.07
1.0	0.11
2.0	0.16
3.0	0.24
4.5	0.44

*Preliminary attempts at the isolation of fumarase.*

Although liver appears to contain much more fumarase than muscle, the enzyme of the aqueous extract of liver is associated with such a large amount of protein, etc. that liver was regarded as an unsuitable source, and rabbit's muscle has been used in these experiments. The minced muscle (back and leg muscles) was first washed by steeping in an equal bulk of water and expressing 16–20 times. The free enzyme is lost in this process but a very large amount of protein, pigment, lactic acid, extractives, etc. are also got rid of. The washed muscle was then steeped for an hour in *M*/15 phosphate and the extract expressed. The process was repeated and the extracts combined.

When 1 % hydrochloric acid was added little by little to this phosphate extract, a precipitate was thrown down which began to coagulate when the  $p_H$  had fallen to 6.5. This precipitate contains the dehydrogenase and was filtered off. The precipitate and filtrate were then rotated in separate bottles with fumarate, the  $p_H$  being readjusted to 7.2 in both cases. The precipitate did not contain any fumarase but the filtrate had a powerful fumarase action. Alcohol and acetone added to phosphate extracts at the ordinary temperature gave inactive precipitates. When the phosphate extract is saturated with ammonium sulphate, however, a very active precipitate is obtained. This



precipitate may be dissolved in a little water and dialysed to remove most of the inorganic salts. As dialysis proceeds, some of the material reprecipitates. The contents of the sac were transferred to a measuring cylinder, the  $p_H$  adjusted to 7.2 and the material divided into two equal portions, one of which was rotated with fumarate and the other with fumarate and the optimal amount of phosphate. Not only did the control show very considerable fumarase activity but phosphate also showed the usual activating effect.

*Similarity of glyoxalase and fumarase.*

There is a close resemblance between the properties of fumarase, which adds on the elements of water to fumaric acid, and glyoxalase which adds on the elements of water to glyoxals, *e.g.* with methylglyoxal giving lactic acid.

Thus, glyoxalase was shown by Dakin and Dudley [1913, 1] to be sufficiently finely dispersed to be filtrable through paper without very great loss and fumarase appears to be similarly finely dispersed. Both enzymes are destroyed at some temperature above 48° but below 60°, and an active extract of both may be obtained by precipitating with ammonium sulphate, dissolving in water and dialysing. Both enzymes are eluted from rabbit's muscle by alkaline fluids and are more stable in alkaline solution. Moreover both enzymes are very widely and similarly distributed, the only difference in distribution so far observed being in the case of yeast. Yeast, according to Dakin and Dudley [1913, 1], contains glyoxalase but fresh samples (exposed for sale) on mixing with water and rotating with fumarate did not appear to contain fumarase. Finally the action of pancreatic juice, which contains antiglyoxalase, [Dakin and Dudley, 1913, 2] on the action of fumarase was investigated. The action of fumarase in an aqueous extract of muscle was inhibited but only to a very slight extent, which did not appear comparable with the inhibition of glyoxalase. This is being further investigated.

SUMMARY.

1. The fumarase activity of rabbit's liver pulp is about eight times that of the same moist weight of muscle pulp but this must only be regarded as indicating that liver pulp contains eight times as much free or active enzyme as muscle.

2. An amount of fumarase is removed from muscle and from liver pulp by steeping eight times in an equal weight of water or 0.25 % saline approximately equal in activity in each case to the activity of the tissue pulp itself. Some succinodehydrogenase, which together with fumarase forms the succinoxidase system, is possibly also removed by washing but this could not be detected by the polarimetric method since the dehydrogenase becomes inactivated almost immediately by the gentle rotation necessary for oxygenation.

3. After the amount of enzyme extracted by this process of washing has become very small, steeping the tissue residue in  $M/15$  phosphate or in a

bicarbonate-carbonate buffer solution of the same  $p_H$  (9) and expressing liberates from the muscle residue an amount of fumarase having about six times the activity of the original pulp, whilst from the liver residue only a small amount is obtained. The total fumarase activity of the aqueous and phosphate extracts of 50 g. rabbit's liver is approximately equal to the total activity of the corresponding extracts of 150 g. of rabbit's muscle.

4. Phosphate not only clutes fumarase in virtue of its alkalinity but also activates it, the optimal phosphate concentration at  $p_H$  7.2 being 0.052 M. Phosphate does not appear to activate succinodehydrogenase in the same way as fumarase. Phosphate activates the fumarase of muscle and liver suspensions, of aqueous extracts of these and also of the enzyme preparation obtained by precipitating with ammonium sulphate and removing the salt by dialysis.

5. The temperature coefficient for the fumarase activity of liver pulp is normal (2.24) but of muscle pulp is very much lower (1.4). The activity of muscle pulp on reducing the temperature from 39° to 19° only falls to one-half. The coefficient for the activity of an aqueous extract of muscle is nearer normal.

6. Sodium chloride inhibits the action of succinodehydrogenase considerably, but of fumarase only slightly. Sodium fluoride inhibits the action of fumarase considerably.

7. The presence of fumarase has been detected in invertebrate muscle (*Pecten maximus*, *Anodonta cygnea*) but not in baker's yeast. It was present in the corpuscles but absent from the serum of fresh defibrinated blood.

8. A very active fumarase solution is obtained by precipitating the phosphate extract of washed muscle with ammonium sulphate, filtering and dialysing the solution of the precipitate.

In conclusion, the author desires to thank Prof. H. S. Raper for his interest, helpful suggestions and criticism throughout this work.

#### REFERENCES.

- Anderson (1927). *Skand. Arch. Physiol.* **52**, 187.  
Batelli and Stern (1911). *Biochem. Z.* **30**, 172.  
Boyland (1928). *Biochem. J.* **22**, 362.  
Clutterbuck (1927). *Biochem. J.* **21**, 512.  
Dakin (1922). *J. Biol. Chem.* **52**, 183.  
Dakin and Dudley (1913, 1). *J. Biol. Chem.* **14**, 423.  
——— (1913, 2). *J. Biol. Chem.* **15**, 463.  
Einbeck (1914). *Z. physiol. Chem.* **90**, 301.  
——— (1919). *Biochem. Z.* **95**, 296.  
Fischer (1927). *Ber. deutsch. chem. Ges.* **60**, 2257.  
Grönwall (1923). *Skand. Arch. Physiol.* **44**, 200.  
Hahn and Haarmann (1927). *Z. Biol.* **86**, 523.  
——— (1928). *Z. Biol.* **87**, 107.  
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.  
Platt and Dawson (1926). *Biochem. J.* **19**, 860.  
Svensson (1923). *Skand. Arch. Physiol.* **44**, 306.  
Sahlin (1924). *Skand. Arch. Physiol.* **46**, 64.  
Thunberg (1909). *Skand. Arch. Physiol.* **22**, 430.  
Widmark (1918). *Uppsala Åkare Förenings Fordhandlingar, Ny följd.* **33**, 134.

# CXLIX. A MICROMETHOD FOR THE DETERMINATION OF THE FATS AND LIPINS OF BLOOD.

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THE chief micromethods which have been employed for the estimation of the fats and lipins are the oxidation method of Bang [1918] and Bang and Blix [1927], the nephelometric method [Bloor *et al.*, 1922], and the microtitration method [Stewart and White, 1925]. Bang's method has been recently modified by Bloor [1928]. A large number of papers has been published dealing with modifications of Bang's method. The chief references to the literature are given by Leites [1927].

The technique adopted in the following method is based on the principle of saponifying all compounds in the blood, which contain fatty acids, and of subsequently determining the amount of fatty acids isolated from the products of saponification. The advantages of this procedure are discussed fully by Leathes and Raper [1925], who also give references to the literature.

The original object of the following investigation was to apply Strebinger's [1919] method of oxidation to the estimation of the fats and lipins of blood. Strebinger has proved that the method gives accurate results with chemically pure substances; but, in the course of my own work, the method was found to be a laborious and exacting one when applied to the estimation of the fats and lipins in blood. All possibility of accidental contamination with any organic material, such as fibres of filter paper must be excluded. Washed asbestos, or Jena glass tubes with filtering discs of fritted glass of porosity < 7 had to be used instead of paper. The method is therefore not well adapted for routine work.

A colorimetric method was therefore tried. This method is based on the formation of a coloured salt of the isolated fatty acids of the blood with the base of a dye-stuff. A number of basic dyes belonging to the triphenylmethane series was tried for this purpose. The base of malachite green has the advantage of being colourless in solution, while its salts are green, so that there is only one colour change involved in salt formation; but the interaction of the base with fatty acids is a slow process even when hastened by heat. Ultimately the base of Nile-blue hydrochloride, first used by Lorrain Smith [1908, 1911] for staining neutral fats and fatty acids, was found to give the best results. The colour change due to salt formation with fatty acid occurs rapidly.

## TECHNIQUE OF METHOD.

The Nile-blue hydrochloride used was extracted with ether and with xylene for 12 hours at room temperature. These solvents acquired only a faint red colour with yellow fluorescence; the oxazine dye therefore contained mere traces of the oxazone [Thorpe, 1907]. The 0.16 g. of Nile-blue hydrochloride was then dissolved in alcohol in a 500 cc. volumetric flask. Excess of silver oxide precipitated from 0.25 g. of silver nitrate by the addition of caustic alkali was washed alkali-free with water, dehydrated with absolute alcohol, and finally added as a suspension in alcohol to the solution of the dye-stuff. The solution was then made up to 500 cc. with redistilled absolute alcohol, thoroughly shaken and allowed to stand in the dark at room temperature until all the Nile-blue hydrochloride had been decomposed, and the solution had acquired the pure red colour of the free base. The solution was then filtered from silver chloride and excess of silver oxide through a Jena glass filter tube with fritted glass disc of porosity 5-7. When filtered through paper part of the dye is adsorbed, forming the blue coloured salt. The solution had a pure red colour, and no yellow fluorescence could be detected. A solution of suitable concentration to serve as an indicator for the estimation of the fatty acids isolated from about 0.1 cc. of blood was obtained by diluting part of this filtrate 1 in 10 with pure absolute alcohol. The diluted solution was stored in a brown bottle in the dark, and was found to retain its colour unaltered for several months, no yellow fluorescence being detectable. Since the molecular weight of Nile-blue A ( $C_{20}H_{20}(ON_3Cl)$ ) is 353, the diluted alcoholic solution of the dye base is less than 0.0001 *M*.

*Estimation of total fatty acid content of blood.*

1. Bloor, Pelkan and Allen's method [1922] was adopted for the extraction of the fats and lipins of blood. 2 cc. portions of the alcohol-ether filtrate (≈ 0.1 cc. blood) were measured from a 10 cc. burette graduated in 0.02 cc. into test-tubes of about  $\frac{3}{4}$  inch diameter. 0.5 cc. of 0.1 *N* alcoholic potash was added to each test-tube, the test-tubes were immersed in a beaker of water heated on an electric hot plate and the contents evaporated to dryness. The tubes were then placed in a drying oven at 80–85° for half an hour to remove traces of alcohol. The dry saponified residue was rendered acid with 0.1 cc. *M* phosphoric acid, care being taken in delivering the acid from a microburette that no drops were left adherent to the upper parts of the test-tubes. The tubes were then rotated so that the phosphoric acid was brought into contact with the whole surface of the dry residue, and were placed in a drying oven at 80–85° until the solution in each had been reduced in volume to a drop. 10 cc. of pure light petroleum of boiling-point 50–60° (redistilled from 30 % aqueous caustic potash) were added to extract the fatty acids and the solution was heated to the boiling-point, allowed to cool, and filtered through fat-free filter paper of 5.5 cm. diameter into test-tubes of accurately measured bore.

The tubes used were of resistance glass, and of 17 mm. bore as measured by a graduated cone reading to 0.1 mm. The acid residue was washed thrice with about 2 cc. portions of light petroleum, and the washings were filtered into these test-tubes. Care must be taken that none of the aqueous solution of mineral acid is transferred. The tubes containing the filtrates were then immersed in a beaker of water heated on an electric hot plate. The initial temperature of the bath was about 55° and was allowed to rise gradually to 75° in order to prevent loss of solution through bumping. When the greater part of the light petroleum had been driven off, the test-tubes were placed in a drying oven at 80° for about 30 minutes to remove the last traces of solvent. The tubes were then allowed to cool, and 10 cc. (measured from a microburette graduated in 0.02 cc.) of the dilute solution of the base of Nile-blue were added to each. The solutions were thoroughly shaken, and the tubes stoppered with corks previously washed with redistilled alcohol.

*Preparation of standards for comparison.* Pure palmitic acid (Kahlbaum), m.p. 62.5° (uncorrected), was recrystallised from light petroleum. 0.0641 g. of the recrystallised acid was placed in a 250 cc. flask, dissolved in light petroleum and made up to 250 cc., the solution being 0.001 *M*. Part of this solution was placed in a microburette with stoppered reservoir. The burettes used were either 2 cc., graduated in 0.01 cc., or 10 cc., graduated in 0.02 cc. Seven numbered test-tubes of uniform bore (17 mm.) were placed in a stand, 0.48 cc. of 0.001 *M* palmitic acid was measured into test-tube (1), and then successively (2) 0.58, (3) 0.69, (4) 0.83, (5) 1.0, (6) 1.20, (7) 1.44 cc. into the other test-tubes, the quantities being arranged in a geometrical progression with a common ratio of 1.20. Palmitic acid being a weak acid only converts a fraction of the dye base into the blue coloured cations of the salt. If necessary, the series of tubes may be extended to nine with a progressive increase in the amount of the blue coloured fraction of the dye. The solutions were evaporated to dryness in the water-bath, the last traces of light petroleum being removed by heating the tubes in a drying oven at 80°. 10 cc. of the alcoholic solution of the Nile-blue base were next added to each tube. The test-tubes were then closed with corks previously washed with redistilled alcohol, and sealed with paraffin wax. Their contents were thoroughly mixed and the solutions allowed to stand for ten minutes. If kept in the dark the standard solutions retain their colours unaltered for several days. The colours of the solutions in the test-tubes containing the fatty acids isolated from blood were then compared with those of the standard series. The following concrete example may be given. The solution of the fatty acids isolated from 0.1 cc. of blood was found to have a colour intermediate between tubes (4) and (5) of the standard series. 0.1 cc. blood therefore contained more than 0.00083 and less than 0.001 millimol. fatty acid. An approximate estimate of the fatty acid in the blood was thus obtained with an error of less than 20 %. A more accurate result may be obtained by preparing another standard series of tubes with quantities of palmitic acid in a geometrical progression with a smaller common ratio such

as 1.05 covering the range between 0.00083 and 0.001 millimol. A more convenient but somewhat less accurate method is to add the alcoholic solution of Nile-blue base from a 10 cc. microburette to the tube containing the fatty acid from blood until the colour of the solution in it matches with no. 4 of the standard series. If  $y$  be the total volume in cc. of the diluted solution, then the quantity of fatty acid in it is  $\frac{0.00083 \times y}{10}$  millimol. The results of the analyses given later are stated as millimol. fatty acid per 100 cc. or, in the case of those obtained by Bang's procedure, per 100 g. blood. The results may also be stated in terms of any of the higher fatty acids, palmitic, oleic, or stearic, by multiplying the result in millimols. by the factor 0.256, 0.282 or 0.281. The total fatty acid content of 100 cc. of defibrinated ox blood was thus found to be 0.83 (4 estimations), 0.82 (3 estimations), 0.80 (2 estimations) millimol. of fatty acid. When Bloor's method is applied to whole blood, Bloor [1928] states that the extraction of fats and lipins may not be quite complete. Hence the foregoing results are possibly too low, but are in good agreement with one another in view of the small quantities of blood analysed.

II. In a second series of experiments Bang's method of weighing the blood for analysis was adopted. About 100 mg. (98.5 to 126) of defibrinated ox or sheep blood were placed on a weighed slip of fat-free absorbent paper, and the weight of blood determined by difference, a torsion balance reading to 1.0 mg. being used. The pieces of paper with absorbed blood were then dried in the air, placed in numbered test-tubes and stored in an evacuated desiccator over calcium chloride.

### 1. *Determination of the total fatty acid content of the blood.*

This estimation, which gives the total fatty acids present as neutral fats, soaps and lipins, was carried out in the following way. The weighed slips of paper with dried blood were extracted with 10 cc. of boiling absolute alcohol. The tubes containing the alcoholic extracts were allowed to stand overnight. The alcoholic solutions were again boiled, cooled and transferred to a second set of test-tubes. The paper slips with blood were then washed once with alcohol and twice with ethyl ether, and the washings transferred to the second series of tubes. 0.5 cc. 0.1 *N* alcoholic potash was added to each, the tubes were immersed in water in a beaker, heated on an electric hot plate, and their contents evaporated to dryness. The saponified material was then heated in an electric drying oven at 85° for at least 30 minutes to remove the residual alcohol. The higher fatty acids were set free by the addition of 0.1 cc. of *M* phosphoric acid to the saponified residue. The other details of the procedure were the same as those already described in the method for analysing the alcohol-ether filtrate used in Bloor's method.

In order to ensure that all solvents and papers used were free from fats, a blank experiment was carried out with a negative result, the red colour of the alcoholic solution of the Nile-blue base remaining unaltered.

## 2. *Estimation of fatty acids derived from neutral fats.*

The neutral fats were extracted from the dried blood with light petroleum. Bang [1918] obtained low values for the neutral fat by the oxidation method, varying from 0.01 to 0.05 % in human blood of normal individuals on a mixed diet. For this reason larger amounts of blood were used. The first estimation was carried out with 0.211 g. of ox blood absorbed on two slips of fat-free paper. For the two estimations of neutral fat in sheep's blood, the quantities taken for analysis were 0.363 g. and 0.337 g., the blood samples being absorbed on three slips of paper. The dried blood was boiled in each case with 10 cc. of light petroleum, and the extracts were allowed to stand at room temperature in stoppered tubes for at least 12 hours. The extracts were filtered, and evaporated to dryness. The residues were then saponified with 0.5 cc. of 0.1 *N* alcoholic caustic potash, and 3 cc. of alcohol. The remaining details of the technique were the same as those already described for the estimation of the total fatty acids. Since the concentration of fatty acids isolated from the neutral fat of blood is normally much lower than that of any of the standard solutions of palmitic acid, the quantity *y* cc. of the dilute alcoholic solution of Nile-blue base finally added to obtain matching of the colours was always much less than 10 cc.

In view of the possibility that the low values for fatty acid of neutral fat might be due to incomplete extraction by the light petroleum, the following method of successive extraction with alcohol and light petroleum was tried. The samples of dried blood were first extracted with absolute alcohol, and the residues left after evaporation of the alcohol were extracted with light petroleum. The light petroleum extract was then filtered, and treated in the way already described. The values given in the table are much higher than those obtained by direct extraction with light petroleum. A preliminary extraction with alcohol thus appears to set free part of the neutral fat, or to render it accessible to the solvent action of light petroleum.

A few experiments were also carried out with acetone as solvent. The acetone was allowed to act on the dried blood at room temperature for 24 hours.

Some of the values obtained by the foregoing method are recorded in the following table, the same sample of ox or sheep blood being used for all the determinations.

### *Millimol. fatty acid per 100 g. blood.*

Blood	Alcoholic extract	Light petroleum (direct extraction)	Alcohol and light petroleum extraction	Acetone
Ox	0.83	0.07	0.42	0.49
	0.81	—	0.43	—
	0.82	—	—	—
Sheep	0.88	0.12	0.45	0.52
	0.90	0.12	0.43	0.53
	0.91	—	0.44	0.50
	0.88	—	0.43	—

*Purification of solvents.*

All solvents must be completely freed from acids and fats. Light petroleum (B.P. 50–60°) ("analytical reagent and free from aromatic hydrocarbons") was thoroughly washed with a concentrated aqueous solution of potassium hydroxide, and distilled from this solution in an all-glass still. The absolute alcohol was redistilled from calcium oxide. The ethyl ether was washed with an aqueous solution of caustic alkali, freed from aldehyde and acids with solid potassium hydroxide and finely powdered potassium permanganate, decanted into a second bottle and dehydrated with calcium chloride. It was finally distilled. The acetone was washed for several days with a saturated solution of potassium carbonate, and distilled. All distillations were carried out in all-glass stills, with ground in glass connections.

Each solvent was then tested in the following way. About 20 cc. were evaporated to dryness in a test-tube, and 10 cc. of the alcoholic solution of Nile-blue base were added. The red colour of the solution of the base remained unaltered. This simple method enables one to ascertain whether any given solvent can be used with safety.

All glassware was washed with a solution of chromic acid in concentrated sulphuric acid. Washing with potassium permanganate solution (about 2 %), followed by the removal of any adherent manganese dioxide with hydrochloric acid, was found later to be an equally effective and more convenient method [Horst, 1921].

## DISCUSSION.

The present communication deals only with the technical details of the method. Any discussion of the significance of the experimental figures would be premature, until a much larger number of comparative analyses has been completed. The writer intends to apply the method to the investigation of human blood.

## SUMMARY.

1. A method for the estimation of the total fatty acids in small quantities of blood has been described.
2. By selective extraction of the dried blood with different solvents, the fatty acid content of some of the components in the mixture of substances extractable by alcohol can be determined.

## REFERENCES.

- Bang (1918). *Biochem. Z.* **91**, 104 and 235.  
 Bang and Blix (1927). *Mikromethoden zur Blutuntersuchung*.  
 Bloor (1928). *J. Biol. Chem.* **77**, 53.  
 Bloor, Pelkan and Allen (1922). *J. Biol. Chem.* **52**, 191.  
 Horst (1921). *Chem. Ztg.* **45**, 604.  
 Leathes and Raper (1925). *The fats*, p. 58 (London).  
 Leiteš (1927). *Biochem. Z.* **184**, 298.  
 Lorrain Smith (1908). *J. Path. Bact.* **12**, 1.  
 — (1911). *J. Path. Bact.* **15**, 53.  
 Stewart and White (1925). *Biochem. J.* **19**, 841.  
 Strebingner (1919). *Z. anal. Chem.* **58**, 97.  
 Thorpe (1907). *J. Chem. Soc.* **91**, 324.



## CL. PROTEIN AND VITAMIN B.

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BOTH protein and vitamin B<sup>1</sup> are accepted as essential constituents of the diet, but the amount of protein which can be metabolised without harmful results is a question on which there is a variety of opinions. For some time it was thought that a high protein diet produced nephritis, but more recent work seems to show that an animal can tolerate large proportions of protein in its diet. Osborne and Mendel [1924] succeeded in feeding rats on a diet containing as much as 80 % caseinogen, while Osborne, Mendel, Park and Winternitz [1926] gave a diet consisting of 95 % caseinogen and found that the rats appeared fit and well. These observers obtained good growth to adult size with rats on high protein diets, but noticed hypertrophy of the kidneys, when the protein comprised one-third or more of the total ration.

Addis, MacKay and MacKay [1926] find that rats can "live for a third of their life-span on a diet very high in casein (70 %) or with added cystine, and yet escape any damage to their kidneys." Jackson and Riggs [1926] fed rats on diets containing 76 % caseinogen, and 20 % caseinogen + 56 % egg-albumin; they found hypertrophy of the kidneys, but no nephritis. The success of these various workers in feeding high protein diets suggests that the failure of others with similar diets is due, not to the high proportion of protein itself, but rather to a deficiency of some other dietary factor or to a lack of balance between some of the constituents. There is evidence that vitamin B is closely related with protein metabolism.

Hartwell [1922, 1, 2, 1924, 1925, 2] has shown that there is a definite relation between the amount of vitamin B and protein in the diet of the lactating rat. Reader and Drummond [1925] found that rats fed on a diet containing 20 % caseinogen grew to adult size, and attained a weight of over 300 g., but when the protein was raised to 45 % none of the rats reached 250 g., while on 90 % of caseinogen no rat grew to 150 g. The animals appeared to be in good health, and *post mortem* examinations showed nothing abnormal, except hypertrophy of the kidneys. In a later paper [1926] these observers confirmed this result, and further found that in order to obtain good growth on such a diet the proportion of yeast extract (vitamin B) must be raised so

<sup>1</sup> By the term vitamin B is meant the original water-soluble B which has now been shown to consist of at least two factors.

that "the ratio of protein to yeast has a value 5 or under." Thus there is evidence that for maximum growth the vitamin B of the diet must be increased when the protein is increased. The proteins used in experiments quoted above were caseinogen, and a mixture of caseinogen and egg-albumin. Bond [1922] obtained good growth in rats on a diet containing 30 % of egg-albumin and 5 % marmite. This relation between protein *qua* protein and vitamin B suggests that there may be some proteins which will require more vitamin B than others for their metabolism, since proteins vary considerably in their composition. Experiments to be described in this paper demonstrate that for normal metabolism edestin requires far more vitamin B than does caseinogen or egg-albumin.

# EXPERIMENTAL.

Piebald rats bred in the laboratory were used. Twelve rats, 6 ♀ and 6 ♂, were used in each experiment, except where otherwise stated, and each experiment was started when the rats weighed approximately 40 g., except Exp. 11. The basal diet consisted of

- 20 g. edestin<sup>1</sup>.
- 64 g. potato starch.
- 12 g. butter.
- 4 g. salt mixture [Hartwell, 1922, 1].
- 300 cc. distilled water.

The food was made freshly every day except Sunday, the "cooked" method being used [Hartwell, 1925, 1]. The same sample of marmite was used throughout. The rats were allowed food *ad lib.*, and distilled water to drink. They were weighed daily for the first 4 weeks, and afterwards the weighing was omitted on Saturdays and Sundays. The animals were not kept on screens (except for part of Exp. 9) because this work was started in 1925 when the necessity for such a precaution was not realised. The floor of the cage was covered with sawdust to which the animals had access. All the experiments to be described in this paper were made under the same conditions and are therefore comparable. The growth curves are given in Fig. 1.

*Exp. 1.* Basal diet + 2 g. marmite.

After about a week several of the rats became weak and were obviously ill, 5 of them died on the 13th day, having shown a previous loss in weight. The remaining 7 were killed and *post mortem* examination showed only one to be normal, while the other 6 had kidneys of a deep purple colour and gorged with blood. (The histological details will be described in a later paper.)

*Exp. 2.* Basal diet + 2 g. marmite.

The results described above were so striking that it was thought advisable to repeat the experiment. Accordingly 12 more young rats were fed on a similar diet, and 6 died by the 14th day; 4 more were killed and each showed the typical purple kidneys. The remaining 2 were kept for 9 weeks on the diet

<sup>1</sup> The edestin used in these experiments was made by Messrs E. T. Pearson and Co. Ltd.

and were then killed. Their kidneys were slightly yellowish, but otherwise normal.

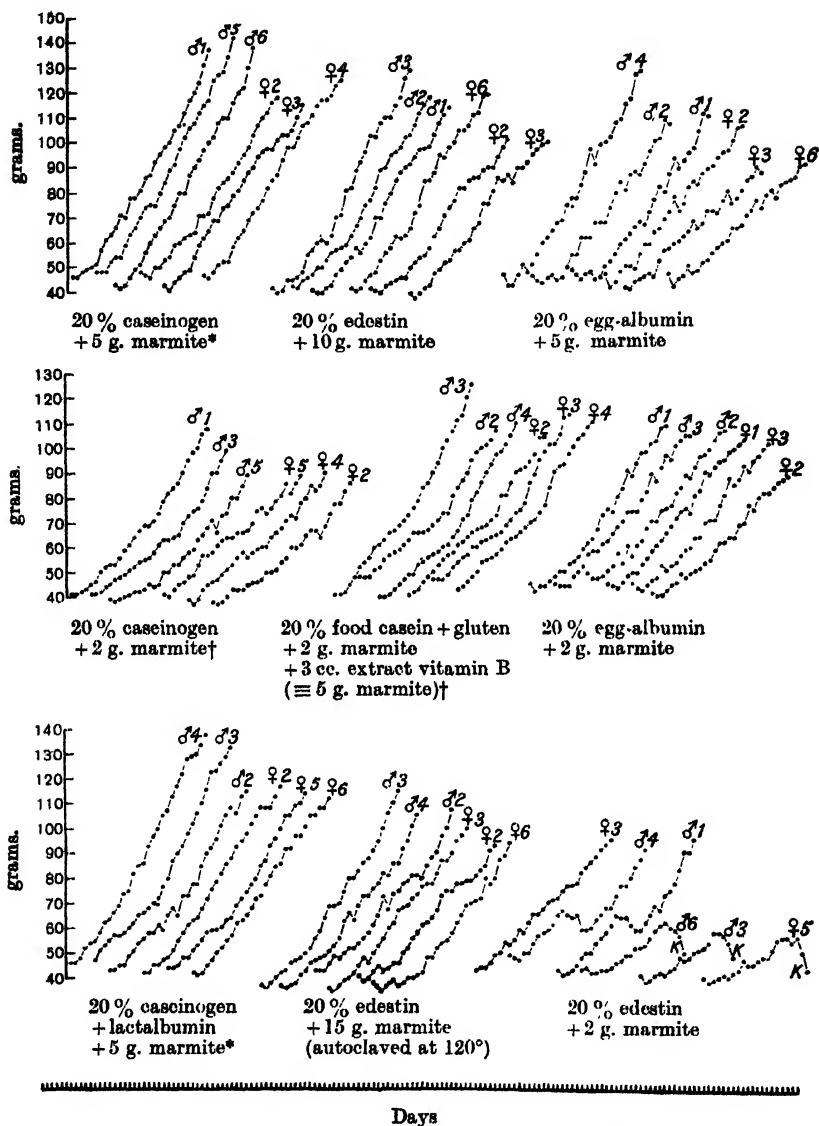


Fig. 1. Growth curves of rats on synthetic diets.

\* Diets described by Hartwell [1927].

† Rat "killed." " [1928].

### Exp. 3. Basal diet + 2 g. marmite.

It was thought that the above results might be due to some alkaloid adhering to the edestin, and therefore a new sample was prepared by the

method of Osborne and Mendel [1894]. The results appeared little better, only 2 rats out of 12 showing normal kidneys after 5 weeks on the diet. Three animals died in 14 days and one was killed after a bad loss in weight; this rat had the typical purple kidneys.

*Exp. 4. Basal diet + 2 g. marmite.*

In the next experiment the edestin used was subjected to several hours' further washing in cold water, in the hopes of removing the hypothetical impurity. Two rats died in 14 days and 4 others which showed the typical loss in weight were killed; all 4 had purple kidneys. After 4 weeks on the diet, the remaining 6 were killed and 5 appeared to be normal. These results were certainly better, but as half the rats developed abnormal kidneys, the edestin could still not be regarded as a satisfactory food protein. It was therefore decided to try edestin which had been extracted with alcohol and if that failed to try extraction with ether.

*Exp. 5. Basal diet + 2 g. marmite.*

The edestin was extracted with 93.58 % ethyl alcohol by weight, the extraction being repeatedly carried on for a period of 3 weeks.

Of the 12 rats used in this experiment, 1 died within a week and 2 more on the 18th day, at which time 4 others which showed loss of weight were killed; all 7 showed the typical purple kidneys. The remaining 5 animals were killed after 5 weeks on the diet, and only one had normal kidneys.

*Exp. 6. Basal diet + 2 g. marmite.*

The edestin used in this experiment was extracted with alcohol as in Exp. 5 and then further extracted with ether repeatedly for a period of 2 weeks.

After 2 weeks on the diet 1 rat was eaten, and therefore 5 others which had lost weight were killed; each had enlarged and purple kidneys. The other 7 rats were kept for  $3\frac{1}{2}$  weeks on the diet and then killed; macroscopically 5 appeared to have normal kidneys.

*Exp. 7. Basal diet + 2 g. marmite + 3 cc. extract containing vitamin B [Hartwell, 1922, 2].*

In view of these results, it seemed unlikely that any poisonous substance adhering to the protein was responsible for the bad effects and therefore it was thought advisable to alter the proportions of other constituents of the diet. To the basal diet + 2 g. marmite were added 3 cc. of an extract containing vitamin B ( $\equiv$  3 g. marmite). On this diet 7 rats died in 14 days, at the end of which time the other 5 were killed, only 2 of them showing normal kidneys.

*Exp. 8. Basal diet + 15 g. marmite.*

18 rats were used in this experiment and 15 g. marmite were given. With this addition to the diet, the growth curves were good and no loss in weight occurred. Six rats, 3 ♀ and 3 ♂ were killed after 2,  $3\frac{1}{2}$  and 5 weeks respectively on the diet, and each animal had normal kidneys, thus it was obvious that the yeast extract had prevented the bad symptoms.

*Exp. 9. Basal diet + 15 g. marmite (modified after 4 weeks).*

Exp. 8 lasted only 5 weeks and it was decided to repeat it and continue

the diet for a longer period in order to see if the improvement were temporary or permanent, since the addition of marmite might have delayed, but not prevented, the kidney symptoms. The basal diet + 15 g. marmite was given for 4 weeks and after this the rats were placed on screens in order that the work might be comparable with another series of experiments. At the same time the basal diet was modified so that the mineral salts were increased, 8 g. instead of 4 g. being used, while the starch was correspondingly lowered to 60 g. instead of 64 g. This modification was introduced because there was some indication in another experiment that the proportion 4 % of mineral salts was rather low for breeding. On this modified diet the rats lived for 12 weeks. All the animals were in excellent condition, their fur was thick and silky and did not come out at any period. The modification in diet and technique introduced at the end of the 4th week made no appreciable difference to the growth curve. During the experiment 5 does each produced 2 litters and the 6th one had 3 fetuses in the uterus when killed. *Post mortem* examination showed that all organs and tissues were macroscopically normal, except for the thyroids which were slightly enlarged.

*Exp. 10.* Basal diet + 10 g. marmite.

An experiment was next made using only 10 g. marmite to see if this quantity were adequate. After 4 weeks on the diet all 12 rats appeared fit and well and their growth curves were good, in fact the animals grew at the same rate as when they received 15 g. marmite. Accordingly 2 ♀ and 2 ♂ were killed and as *post mortem* examination revealed no abnormalities, the other rats were not killed.

*Exp. 11.* Basal diet + 2 g. marmite (older rats).

The next problem was to see if rats of all ages developed kidney symptoms on the edestin diet and therefore an experiment was made using partly grown rats. Six males ranging from 98 to 145 g. and 5 females from 85 to 110 g. were used. From weaning until given the edestin diet, the rats were treated as stock animals, *i.e.* they received a little bread and milk every day and kitchen scraps *ad lib.* After 14 days on the edestin diet, 2 ♀ and 2 ♂ were killed; their kidneys appeared quite normal. After 6 weeks, 2 ♀ and 2 ♂ were killed; and as they also were normal in all respects, the experiment was discontinued. One doe produced a litter of 6 and reared 4 of them (but the marmite was increased during the lactation period). All the rats grew steadily and well, though less rapidly than on a control diet of kitchen scraps.

*Exp. 12.* Basal diet + 15 g. marmite autoclaved at 120° for 4½–5 hours.

(a) This diet was given for 17 days during which time the rats grew well and appeared to be in good condition. Since the loss in weight and death of some animals was usually noticed before the 14th day, it seemed that these rats were safely over the danger period, but 4 were killed to see if the kidneys were abnormal; all 4 however appeared quite normal.

(b) A second experiment was made in which the rats were kept on the basal diet + autoclaved marmite for 6 weeks. During this period the growth

curves were good and steady, no loss in weight being shown. The rats were all in excellent condition at the end of the 6 weeks, therefore the factor in marmite which prevented the kidney symptoms had not been destroyed by the high temperature.

Previous experiments with other proteins have shown good growth with 5 g. or less of marmite in 100 g. dry solid food. Hartwell [1926] obtained steady growth in rats on a diet containing 20 % caseinogen and only 2 g. marmite, and quite good growth with the equivalent of 5 g. marmite and a mixture of "food casein" and gluten. Later [Hartwell, 1927] caseinogen + egg-albumin, and caseinogen + lactalbumin produced satisfactory growth when 5 g. marmite were added to 100 g. solid. Thus it appeared that the metabolism of edestin required more marmite than other proteins and in order to test this hypothesis further, experiments were made with synthetic diets containing 20 % of egg-albumin with 5 g. and 2 g. marmite respectively.

*Exp. 13.* Basal diet in which edestin was replaced by egg-albumin + 5 g. marmite.

Twelve rats were fed on this diet for 4 weeks; growth was good and the animals were fit and well throughout the experiment. *Post mortem* examination of 3 ♀ and 3 ♂ showed normal kidneys.

*Exp. 14.* Basal diet as in *Exp. 13* + 2 g. marmite.

In this experiment 6 ♀ and 5 ♂ rats were used; they grew well during 4 weeks on the diet. Then 2 ♀ and 2 ♂ were killed, but nothing abnormal was seen.

## RESULTS.

### 1. *Edestin experiments.*

A synthetic diet containing 20 % of edestin as the sole protein and 5 g. or less of marmite per 100 g. solid is not a satisfactory food for young rats. Many of the animals die during the first 2 weeks of the experiment, with a characteristic drop in weight before death. Other rats show a similar depression of the weight curve (Fig. 1), but eventually recover. The animals which die become limp and cold, with sunken eyes, but otherwise no external symptoms can be detected. There is no paralysis as with a vitamin B-free diet. *Post mortem* examinations were made on 84 rats and of these only 18 showed normal kidneys. The typical depression in the weight curves is indicative of abnormal kidneys. Partly grown rats can live on a diet of 20 % edestin and 2 g. marmite, and do not develop kidney symptoms.

### 2. *Amount of yeast extract in relation to kind of protein.*

With egg-albumin, the addition of 5 g. marmite instead of 2 g. per 100 g. dry solid made no appreciable difference in the growth curves of the rats, but a corresponding alteration in the caseinogen diet led to a marked improvement in the rate of growth. With edestin on the other hand, 10 g. of marmite were needed to provide for good growth.

### 3. *Rate of growth.*

When egg-albumin was used as the sole protein, the rate of growth was just slower than with either of the other proteins, but the curves were quite good, and the rate of growth steady. With edestin, the growth was equal to that obtained with caseinogen, provided a larger proportion of marmite were added to the basal diet. There appeared to be no difference in the rate of growth of the rats on the edestin diet when they were given 15 g. or 10 g. yeast extract, but with the autoclaved marmite (15 g.) the rate of growth was a little slower.

### COMMENTS.

It seems unlikely that the edestin in itself is responsible for the bad effects obtained in these experiments, because this protein has been used successfully by other nutrition workers for a considerable time. Osborne and Mendel [1916] fed rats on a diet containing 15 % of edestin for 11 weeks, and in other experiments [1919] they used 17.9 % of edestin for a period of 8 weeks. Osborne, Mendel, Park and Winternitz [1926] obtained good growth to adult size with 16 % of edestin, while Osborne and Mendel [1924] were successful in feeding rats on a diet containing as much as 50–55 % edestin. This latter work is very interesting in that Osborne and Mendel state "several attempts to raise animals on fat-free diets containing 95 % of protein of which 90 % was edestin failed because the animals died during the preliminary period of adjustment to the food." Their rats obviously died in a short time, as did mine on the edestin diet, so it is possible that this failure is due to insufficient vitamin B in the diet, especially as they fed 80 % caseinogen successfully. It is not possible to make any definite statement, because there is no way of comparing the respective amounts of vitamin B used, since their yeast (of which 400 mg. were given as a daily ration) may have been relatively stronger or weaker in vitamin B than the marmite used by me.

Exp. 9, in which the rats lived for 16 weeks on a diet containing 20 % of edestin as sole protein, also implies that edestin is a satisfactory constituent, especially as breeding took place at the normal time. It is, however, clear from the experiments described in this paper that for good growth edestin requires more marmite than either caseinogen or egg-albumin. It is possible that some specific amino-acid present to a greater extent in edestin than in other proteins used for feeding experiments is responsible for the amount of marmite required. That there are obvious differences in the metabolism of amino-acids has been shown by Seth and Luck [1925] who found that in the case of histidine there is no increase in the urea content of blood even after 6 hours, while other amino-acids cause appreciable urea production. At the present time one cannot make more than a tentative suggestion on this point, because the analyses of proteins in respect of amino-acids vary so considerably. Since marmite is used in synthetic diets solely as a source of vitamin B, it

can be assumed that more vitamin B is required for the metabolism of edestin, and also, as marmite autoclaved at 120° for 4½–5 hours was equally satisfactory in preventing the kidney symptoms, the antineuritic substance [Chick and Roscoe, 1927] is obviously not the important factor in this case. Presumably the rats obtained sufficient antineuritic substance from excreta to which they had access, or from some constituents of the diet (the butter, or protein which was not pure). It is extraordinary that the older rats did well when only 2 g. marmite were given, and that no kidney abnormalities resulted, especially in view of the work of Osborne and Mendel [1922] demonstrating that the amount of vitamin B required by the rat is proportional to the weight and age of the animal. No doubt when more is known of the complex nature of vitamin B, these discrepancies will disappear, for we are dealing with several factors, not yet completely separated. From the experiments described in this paper the kidney symptoms are undoubtedly characteristic of young rats, possibly analogous to the development of rickets, which disease is only met in young animals.

These experiments support the view that there is a definite relation between the protein and vitamin B of the diet, and further suggest that different proteins may require various amounts of vitamin B in order that they may be properly metabolised. Egg-albumin appears to require less vitamin B than the other proteins used. This may be correlated with the fact that egg-albumin provides for slower growth and therefore a lower proportion of marmite is adequate.

#### SUMMARY.

1. On a synthetic diet containing 20 % of edestin and 5 g. yeast extract per 100 g. dry solid, many young rats die, showing typical kidney abnormality; older rats are not adversely affected by the diet.
2. These symptoms can be prevented by the addition of more yeast extract to the diet.
3. The "protective" factor in yeast extract is not destroyed by autoclaving at 120° for 4½–5 hours.
4. Edestin requires more yeast extract than either caseinogen or egg-albumin for normal metabolism in young growing rats.

I wish to thank Mr Clark and Mr Faraday of Messrs E. T. Pearson and Co. Ltd. for their help and co-operation in this work, and also Miss W. M. Clifford for making the *post mortem* examinations.

The expenses of this research were defrayed by a grant from the Medical Research Council.



## REFERENCES.

- Addis, MacKay and MacKay (1926). *J. Biol. Chem.* **71**, 139.  
Bond (1922). *Biochem. J.* **16**, 479.  
Chick and Roscoe (1927). *Biochem. J.* **21**, 698.  
Hartwell (1922, 1). *Biochem. J.* **16**, 78.  
—— (1922, 2). *Biochem. J.* **16**, 825.  
—— (1924). *Biochem. J.* **18**, 785.  
—— (1925, 1). *Biochem. J.* **19**, 729.  
—— (1925, 2). *Biochem. J.* **19**, 1075.  
—— (1926). *Biochem. J.* **20**, 1273.  
—— (1927). *Biochem. J.* **21**, 1076.  
Jackson and Riggs (1926). *J. Biol. Chem.* **67**, 101.  
Osborne and Mendel (1894). *J. Amer. Chem. Soc.* **16**, 778.  
—— ——— (1916). *J. Biol. Chem.* **26**, 1.  
—— ——— (1919). *J. Biol. Chem.* **37**, 223.  
—— ——— (1922). *J. Biol. Chem.* **54**, 739.  
—— ——— (1924). *J. Biol. Chem.* **59**, 13.  
Osborne, Mendel, Park and Winternitz (1926). *J. Biol. Chem.* **71**, 317.  
Reader and Drummond (1925). *J. Physiol.* **59**, 472.  
—— ——— (1926). *Biochem. J.* **20**, 1256.  
Seth and Luck (1925). *Biochem. J.* **19**, 366.

# CLI. THE MINIMUM AMOUNT OF VITAMIN D REQUIRED FOR A POSITIVE ANTIRACHITIC EFFECT IN THE "LINE" TEST.

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*(Received July 30th, 1928.)*

FOSBINDER, Daniells and Steenbock [1928] made an attempt to determine quantitatively the amount of energy necessary to secure a demonstrable deposition of calcium in the bones of a rachitic rat. Using a monochromator and taking infinite pains to measure the energy transmitted from the radiation of  $265\mu\mu$  wave-length from a mercury vapour lamp to a "pure" sample of cholesterol, they found that the shortest time of this treatment needed to produce enough vitamin D to give what they term a positive antirachitic action in their "line" test, was 22.5 seconds. The energy absorbed by the "cholesterol" during this time was 234 ergs. The number of quanta absorbed was calculated as  $3.2 \times 10^{13}$ . Applying Einstein's law of photochemistry, this would imply that  $3.2 \times 10^{13}$  molecules of vitamin D had been synthesised during the exposure of 22.5 seconds. The number of gram-molecules is then  $5 \times 10^{-11}$  and assuming the molecular weight of the antirachitic material to be essentially the same as that of cholesterol (385) the weight of vitamin D generated in this experiment was  $5 \times 10^{-11} \times 385 = 2 \times 10^{-8}$  g. (It should be noted that this was the whole dose during the 10 days' test period, not a daily dose.) The American workers then prophesied that when pure vitamin D was obtainable this would be found to be approximately the amount required to give a positive result in the "line" test for the antirachitic factor. The writer has obtained results with a sample of irradiated ergosterol which appear to prove this prophecy to have been singularly correct.

The solution of irradiated ergosterol used in the Society's laboratory as a standard preparation of vitamin D was prepared by Mr T. A. Webster at the National Institute for Medical Research under conditions calculated to generate the maximum antirachitic activity in it. Its potency was determined by testing on rats rendered rachitic by the method of Steenbock and Black [1925]. The writer had had the privilege of 10 months' experience of this test under Prof. Steenbock's tuition, so that her standards and assays may fairly be considered comparable with his.

The result of the assay may be summarised thus:

Daily dose of irradiated ergosterol mg.	Degree of calcification in Steenbock's terms of assay
0.0	—
0.000005	Few foci
0.00001	+ (a thin line)
0.00002	+ + (a thicker line)
0.00005	+ + + (a thicker line still)
0.0001	+ + + + (complete healing)

Where Steenbock classified degrees of calcification as "negative," "positive," and "very positive," as he has done in his latest paper, his "positive" result may be considered equal to a + or + + of the above classification, preferably the latter.

The daily dose that gave this positive result was  $2 \times 10^{-5}$  mg., so that the amount consumed in 10 days was  $2 \times 10^{-7}$  g.

Rosenheim and Webster [1927] state that maximum antirachitic activity is generated in ergosterol by their method of irradiation after 30 minutes and that at this time they can recover 90 % of unchanged ergosterol. Assuming that some of the vitamin D formed during the first 30 minutes of irradiation had been destroyed by the rays that appear to keep formation and destruction balanced during the subsequent 180 minutes, it would appear that rather less than 10 % of the irradiated ergosterol consisted of vitamin D. The amount of actual vitamin D contained in  $2 \times 10^{-7}$  g. of the Society's standard preparation of irradiated ergosterol would according to this be about  $2 \times 10^{-8}$  g. This gives an extraordinarily clear confirmation of the American workers' prophecy that the actual amount of vitamin D required to give a positive reaction in the "line" test would prove to be  $2 \times 10^{-8}$  g. Incidentally, also, there is evidence here of the suitability of the "line" test for demonstrating the presence of even smaller quantities than this of vitamin D.

#### REFERENCES.

- Fosbinder, Daniells and Steenbock (1928). *J. Amer. Chem. Soc.* **50**, 923.  
 Rosenheim and Webster (1927). *Lancet*, ii, 622.  
 Steenbock and Black (1925). *J. Biol. Chem.* **64**, 263.

## CLII. NOTES ON THE IRRADIATION OF ERGOSTEROL.

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It was recorded by Rosenheim and Webster [1927] that the exposure of ergosterol to ultra-violet radiation produced a mixture of substances including vitamin D, and evidence was given suggesting that, for moderate periods of irradiation, the amount of vitamin present in the mixture was nearly independent of the period of irradiation, apparently owing to simultaneous formation and destruction of the vitamin. This work has been continued in search of possible means of isolating vitamin D.

### I. *The effect of using "filtered" light at various temperatures.*

Attempts were made to produce more concentrated preparations of vitamin D by the irradiation of ergosterol by selected portions only of the ultra-violet spectrum. If, as was suggested by Morton, Heilbron and Kamm [1927, 1] and also by Pohl [1926], vitamin D shows absorption chiefly between 230 and 260 $\mu\mu$ , while ergosterol shows it chiefly between 260 and 290 $\mu\mu$ , then the use of a filter cutting off all rays of wave-length shorter than 265 $\mu\mu$  should increase the quantity of vitamin D formed during irradiation by reducing the rate of destruction of the vitamin without seriously affecting that of ergosterol. The use of "vitaglass" for this purpose was suggested by Morton *et al.* in the above paper, and has since been tested by them [1927, 2], and by Rosenheim and Webster, with negative results. Alcoholic cobalt chloride is, however, even more suitable for such a test. A 5 % solution of thickness 10 mm. in a cell with plane polished silica walls, transmits between 20 % and 30 % of radiations between 400 $\mu\mu$  and 280 $\mu\mu$  and about 17 % of 280 $\mu\mu$ . The transmission then falls rapidly to 3 % at 265 $\mu\mu$  and then very rapidly indeed to less than one part in a million at 254 $\mu\mu$ . By multiplying the exposures used without a filter by about 4.5, the use of this filter thus secures an undiminished exposure to radiation above 280 $\mu\mu$  and only slight diminution of radiation from 280 to 270 $\mu\mu$ , with freedom from any disturbance at all by radiation of wave-lengths shorter than 265 $\mu\mu$ . Preliminary trials with this filter showed no effect on the

equilibrium, and, as it seemed possible that temperature changes during radiation were affecting the results, the trials were extended over a wide range of temperature.

### *Experimental details.*

The ergosterol was a highly purified crystalline specimen of M.P. 162° prepared from ergot, presented to Dr Rosenheim by Dr H. D. Jowett of Messrs Burroughs Wellcome and Co. and kindly placed at our disposal by Dr Rosenheim. The solvents used were pure ether dried over sodium, and absolute alcohol, these being chosen as the most stable solvents available without marked ultra-violet absorption. Solutions were freshly made before use, and in these experiments were always of concentration 0.1 g. per litre.

For all except the lowest temperatures the source of light was a K.B.B. silica mercury arc worked at atmospheric pressure with 2.5 amp. and 125 volts across the arc. An opaque screen with a circular hole 5 cm. diameter was placed 5 cm. from the lamp and 10 cm. from the ergosterol solution. This solution was contained in a fused silica cell 10 mm. thick, with sides 2.5 x 2.5 cm. of optically plane polished silica. It was immersed in an outer vessel of fused silica containing a fluid kept at constant temperature and a thermometer. For the tests at + 1° and - 18° an air chamber dried by calcium chloride and fitted with a silica window was attached to the side of the bath facing the lamp to avoid deposition of dew. At each temperature exposures were made both to unfiltered radiation and to radiation filtered through the cobalt chloride filter described above. (This filter was circular in section and 2 inches in diameter.) A uniform exposure of 20 minutes for unfiltered light and 90 minutes for filtered light was given in order to secure equal amounts of radiation of wave-lengths between 280 and 350 $\mu$ .

For the tests made at very low temperatures the above cell was immersed in liquid oxygen or nitrogen and a Hewittic vacuum silica mercury arc was placed horizontally 10 inches above the cell, and run at 3 amp. and 130-140 volts after a preliminary test with a photoelectric cell to show that these conditions gave radiation of intensity roughly similar to that used at other temperatures. No part of the body of the cell was above the surface of the liquid, and no exposure to ultra-violet radiation occurred before complete cooling. Alcoholic solutions only were used at these temperatures since they froze to a transparent glass without separation of solute, in contrast to ethereal solutions which solidified as an opaque crystalline mass. The silica cell was occasionally broken by a sudden shattering of the contents, due apparently to further contraction of the glassy mass after it had become rigid. Unfiltered light only was used, and exposure varied from 5 minutes to 5½ hours. All solutions were photographed through a Hilger quartz spectrograph before and after irradiation.

The following table shows the temperatures used and the fluid surrounding the cell at each temperature.

+ 77.8°	Alcohol (boiling)
+ 30.6°	Ether (boiling)
+ 1°	Water
- 18°	Ether (boiling at low pressure)
- 183° approx.	Liquid oxygen
- 195°     "	"     nitrogen

*Biological testing of antirachitic activity.*

Immediately after irradiation an aliquot part of the fluid to be tested was dissolved in 5 cc. of olive oil and the alcohol or ether removed *in vacuo* at room temperature. This solution was then suitably diluted with olive oil so that the required amount of irradiated ergosterol was contained in 20 mm.<sup>3</sup> of oil.

The test animals were young albino rats of about 40 g. weight and the diet used was the modified diet 84 [Rosenheim and Webster, 1926]. In most cases the protective method was used, the animals being kept on the diet for 21 days, during which period daily doses of the test solutions were given to all except the control animals. At the expiration of this time the animals were killed and the left hind leg was dissected out and photographed with a soft X-ray tube. The limbs from each litter of rats were photographed on one plate, and the degree of rickets in each rat was carefully estimated.

Although litter mates react fairly consistently to rachitic diets and anti-rachitic treatment, different litters show much less consistency. To overcome this variation each litter of rats was divided into two groups; one group received graded doses of the solution under test and the other graded doses of a standard solution. Comparison of corresponding members of the two groups then gave a measure of the activity of the solution under test in terms of the standard.

Each rat received one dose per day, Sundays excepted, administered with Wright's capillary pipette. The use of a standard preparation for the assay of vitamins has also been described by Coward [1928].

The standard solution of irradiated ergosterol was prepared as follows. A 0.1 % solution of ergosterol in dry ether was exposed to the radiations of a K.B.B. silica mercury vapour lamp taking 2.5 amp. at 125 volts for 40 minutes. The ether solution was contained in the standard silica cell 1 cm. thick at a distance from the lamp of 8 inches. After exposure the ether was rapidly removed on the steam-bath in a current of nitrogen and the residue dissolved in olive oil, the concentration being 0.01 %. This solution was kept frozen solid at about 0° and dilutions were made when required. Fresh dilutions were made for every set of experiments, so that no solution was kept at room temperature longer than 3 weeks.

*Results of antirachitic tests.*

All products appeared of approximately equal activity except those obtained in liquid oxygen or nitrogen, which were markedly less active. The results obtained from  $+78^{\circ}$  to  $-18^{\circ}$  are based on tests on about 170 rats, and are probably fairly reliable for this class of work. A difference of 100 % between any two fluids would probably have been evident, and a regular difference of this magnitude between filtered and unfiltered light would be most unlikely to have escaped detection.

The products obtained at  $-183^{\circ}$  and  $-195^{\circ}$  are definitely less active. In a series of trials products have been obtained varying from one-tenth to one-half of the standard strength, but in some series both the antirachitic activity and the spectrographic changes in the solutions are less than in others. The cause of this variability is not yet certain, but may be turbidity of the liquid nitrogen or oxygen, caused by particles of ice with which it is liable to become contaminated.

A further test of the filtered light at  $30^{\circ}$  was made by prolonged irradiation of 0.1 % solutions of ergosterol in ether for periods of 1, 4, and 63 hours respectively.

The first two fluids showed a maximum standard activity, while that given 63 hours' exposure (equivalent to about  $1\frac{1}{2}$  hours' exposure of 0.01 % solution to unfiltered light) was less than one-quarter as active.

It seems reasonable to conclude that the exclusion of wave-lengths shorter than  $265\mu\mu$  does not seriously alter the ratio of rates of production and destruction of vitamin D. This suggests, but does not prove, that vitamin D either has strong absorption for wave-lengths longer than  $265\mu\mu$ , or has not great absorption for wave-lengths of 230 to  $250\mu\mu$  as suggested by previous workers.

The lack of marked effect of changes of temperature between  $+78^{\circ}$  and  $-18^{\circ}$  on the equilibrium, shows that the temperature coefficients of the changes causing production and destruction are not widely different, and the very moderate effect of lowering the temperature to  $-180^{\circ}$  suggests that the temperature coefficient of both reactions is very small, and hence that both reactions are directly photochemical in nature.

## II. *Some properties of the products of irradiation after removal of unchanged ergosterol.*

The products remaining after the removal of unchanged ergosterol from solutions irradiated for short periods only are being investigated. By this means it was hoped to secure potent preparations of vitamin D free from ergosterol and containing a reduced proportion of inactive decomposition products. The technique was as follows. A 0.1 % or 0.15 % solution of ergosterol in alcohol was irradiated for periods of  $\frac{1}{2}$  minute to 20 minutes in a layer 10 mm. thick at 3 to 6 inches from a mercury vapour lamp. The irradiated solution was treated (either with or without previous concentration by evaporation

*in vacuo*) with a 1 % solution of digitonin in 95 % alcohol, using an amount of digitonin equal to 5 times the weight of ergosterol present before irradiation, i.e. an excess of more than 25 % over the quantity theoretically needed for precipitation. Enough water was added to bring the total concentration of water up to 10 %. The mixture was allowed to stand overnight and then filtered. The precipitate of ergosterol digitonide was rejected. The filtrate was in some cases used for spectrographic and biological tests, but always contained small traces of ergosterol. To remove these the solution was evaporated to dryness *in vacuo* at about 40° (thus ensuring the complete precipitation of ergosterol during concentration), and the residue was then extracted with dry ether, in which digitonin and ergosterol digitonide are practically insoluble. The solution was used as such, or evaporated to dryness in order to weigh the residue. All these operations were carried out as far as possible in an atmosphere of nitrogen and at temperatures never exceeding 50°. The application of this process to non-irradiated ergosterol yields only traces of oily substances which are without appreciable absorption.

The products so obtained formed a transparent glassy hard solid of indefinite M.P. beginning about 30°, at times colourless, but more often contaminated with a yellow pigment which appeared during the original solution in alcohol of certain samples of ergosterol [Tanret, 1908]. The product is soluble in approximately its own weight of alcohol at 30°. This is in striking contrast to the small (0.2 %) solubility of ergosterol in alcohol, and is a proof of the efficiency of the process described above for removing ergosterol. The product is also very soluble in ether, chloroform and light petroleum, but insoluble in water. It is moderately soluble in methyl alcohol containing 10 % of water, and this mixture was used in attempts to fractionate the mass. It shows high antirachitic activity. This appears to vary in different samples prepared on different occasions. The highest observed activity appears to have been about twice that of the standard described above. The absorption spectrum varied considerably in different samples, and is discussed in the next section.

### III. *Absorption spectra of products of irradiation.*

Preliminary studies of absorption have been made by a photographic method, using a modification of the technique described by Dobson, Griffith and Harrison [1926]. The results obtained were in general concordant to about 5 %, but difficulty was found in controlling the voltage on the lamp used, and at times a larger error occurred. Greater sources of error were found in the presence of traces of the yellow pigment referred to above, which showed strong absorption between 300 and 400 $\mu$ . For these reasons the curves shown are only rough approximations, and no attempt has been made to locate exactly the heads of the individual bands.

Curve 1 (Fig. 7) and (Plate III, fig. 1) show the absorption of the ergosterol used.



Curve 2 (Fig. 7) and (Plate III, fig. 2) show the absorption of a practically colourless sample of the product obtained after the removal of ergosterol as described above.

Curve 3 (Fig. 7) shows the absorption of another sample of the product.

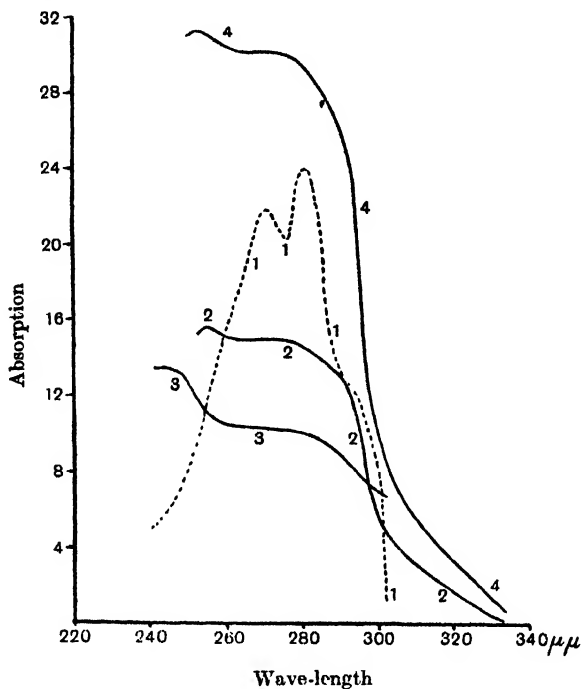


Fig. 7.

These products show strong absorption for wave-lengths shorter than  $290\mu\mu$  and in these samples show no maximum between  $265$  and  $280\mu\mu$  in contrast to ergosterol and to certain other samples of products in which a maximum occurred between  $300$  and  $330\mu\mu$ . The absorption is, however, much greater than that of ergosterol. The above features can be seen by inspection of Plate III, figs. 1 and 2, and are of much interest in conjunction with the following phenomena, which can be observed during the earliest stages of the irradiation of ergosterol.

If a solution of ergosterol be examined after short periods of irradiation, an increase in absorption is noteworthy, in contrast to the decrease recorded by many workers after longer periods of radiation. In a 10 mm. layer of 0.2 % solution this increase is very striking at  $313\mu\mu$  (Plate IV, fig. 3), while in 0.1 % solution it is obvious at  $302\mu\mu$  (Plate IV, fig. 4), and in 0.01 % solution it is evident at  $290$  and  $265\mu\mu$  and visible at  $253\mu\mu$  (Plate IV, fig. 5) after 4 minutes' irradiation. In 0.05 % solution it is just visible in the intervening wave-lengths between  $290$  and  $265\mu\mu$  after about 2 minutes' irradiation. The increase is at its maximum after a period depending on the concentration

of solution as well as on the intensity of radiation. Under our conditions this maximum occurs after about 6 minutes in 0.01 % solution, or an hour in 0.1 % solution (the exact times have not been measured). On further irradiation the absorption decreases steadily to the almost complete disappearance often recorded. In fig. 5 (Plate IV) a marked decrease in absorption is seen after 20 minutes' irradiation.

These changes do not occur if a filter of thick window-glass is interposed between the lamp and solution, but do occur if filters of chlorine or alcoholic cobalt chloride are used, although the latter modifies the changes. They appear unaffected by bubbling either oxygen or nitrogen through the solution during irradiation, or by the use of ether as solvent instead of alcohol. The most simple explanation of them is the hypothesis that ultra-violet irradiation has as an initial (or early) effect the formation from ergosterol of products showing strong absorption between 313 and  $250\mu\mu$ . The intensity of this absorption markedly exceeds that of ergosterol between 320 and  $290\mu\mu$ , and between 265 and  $250\mu\mu$ , and slightly exceeds it between 290 and  $270\mu\mu$ . (We have only worked with a mercury vapour spectrum and so have not excluded the possibility of narrow troughs between bands, in which the absorption may be less than that of ergosterol.) This hypothesis is strongly supported by the absorption of the products described above which remain after the removal of ergosterol from an irradiated solution. For, although these products show an absorption which only exceeds that of ergosterol between 300 and  $330\mu\mu$ , if it is assumed that the products are a pure absorbing substance, it is much more probable that the products are a mixture of absorbing and non-absorbing substances. If such non-absorbing substances form 40 % of the sample whose absorption is given in curve 2 (Fig. 7), the true absorption curve of the absorbing product would be that shown in curve 4 (Fig. 7). Such an absorption would satisfactorily account for the increase in absorption shown in the first few minutes of the irradiation of ergosterol.

#### IV. Possible absorption spectrum of vitamin D.

We have made numerous attempts to find a quantitative relation between the magnitude of the absorption coefficients of the products described above and their antirachitic activity. Owing to the difficulties of such tests, no exact relationship has been proved or disproved, but the following conclusions can be stated.

1. If a 0.1 % solution of ergosterol in alcohol or ether is irradiated at room temperature under the conditions described in Section I, products having an antirachitic activity and absorption of the type described are present to a small extent after 30 seconds' irradiation, and to a marked extent after 1 minute's irradiation.

2. After 10 minutes' irradiation both properties are increased to something like 10 times the former values (Plate IV, fig. 6).

3. Irradiation for periods of between 10 and 60 minutes increases both properties somewhat further, to about 2 or 4 times their value after 10 minutes' irradiation.

4. Thus there is evidence that both antirachitic activity and absorption are produced at approximately the same rate, and that both begin in the earliest stages.

5. If the products of irradiation after removal of ergosterol are exposed to ultra-violet light, both antirachitic activity and absorption decrease, and both disappear almost entirely after between 3 and 5 hours' irradiation under the above conditions.

6. We have not yet succeeded in obtaining a product showing either antirachitic activity without the type of absorption described, or *vice versa*. Each property has always been accompanied by the other. There is thus some reason for thinking that absorption of the type shown in curve 4 (Fig. 7) may be a property of vitamin D. This conclusion is, however, only one of several possible explanations of the observed phenomena, and is not easy to reconcile with the observations of Morton *et al.* or of Pohl [1926]. A more probable explanation, which we hope to test shortly, is that the irradiation of ergosterol produces two substances in succession, of which the first has an absorption maximum at about 280 or 290 $\mu\mu$ , and the second a maximum at about 230 $\mu\mu$ , and that the former is vitamin D. This hypothesis would explain the variable absorption of the products obtained after removing ergosterol from irradiated mixtures, and would also fit the absorption changes found by us in the early stages of the irradiation of ergosterol and the changes found by the other authors quoted in the later stages. On any hypothesis the products of irradiation must show considerable antirachitic activity until some time after the practical disappearance of ergosterol, and this makes the evidence attributing absorption at 240 $\mu\mu$  to vitamin D open to question unless a quantitative relation between such absorption and antirachitic activity can be shown. As arguments suggesting a connection between vitamin D and absorption at 280 $\mu\mu$  may be quoted the very early production of marked antirachitic activity on irradiation of ergosterol, and the fact that in our experiments we have always found marked absorption at 290 and 300 $\mu\mu$  in products showing antirachitic activity.

We desire to express our thanks to Dr G. M. Dobson, F.R.S., for advice on photometry and for the use of his own photometer.

#### REFERENCES.

- Coward (1928). *Quart. J. Pharm.* 1, 27.  
Dobson, Griffith and Harrison (1926). Photographic photometry. (Clarendon Press, Oxford.)  
Morton, Heilbron and Kamm (1927, 1). *J. Chem. Soc.* 2000.  
——— (1927, 2). *Nature*, 120, 617.  
Pohl (1926). *Nachr. Ges. Wiss. Göttingen*, 185.  
Rosenheim and Webster (1926). *Biochem. J.* 20, 537.  
——— (1927). *Lancet*, ii, 622.  
Tanret (1908). *Ann. Chim. Phys.* (8) 15, 313.

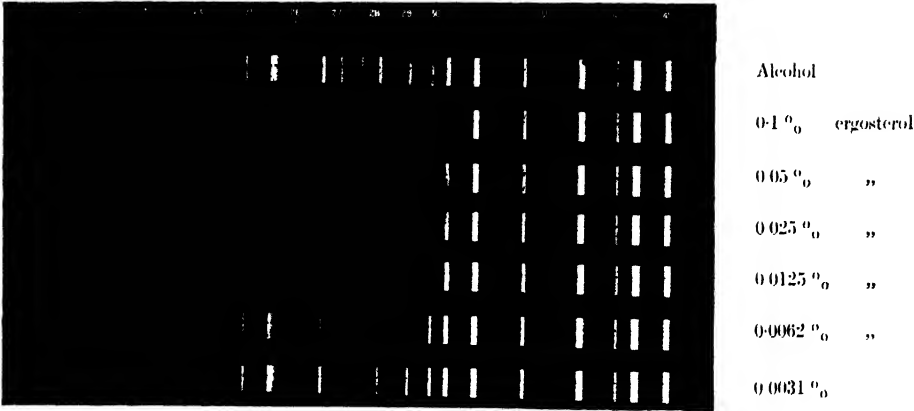


Fig. 1. Ergosterol in alcohol.

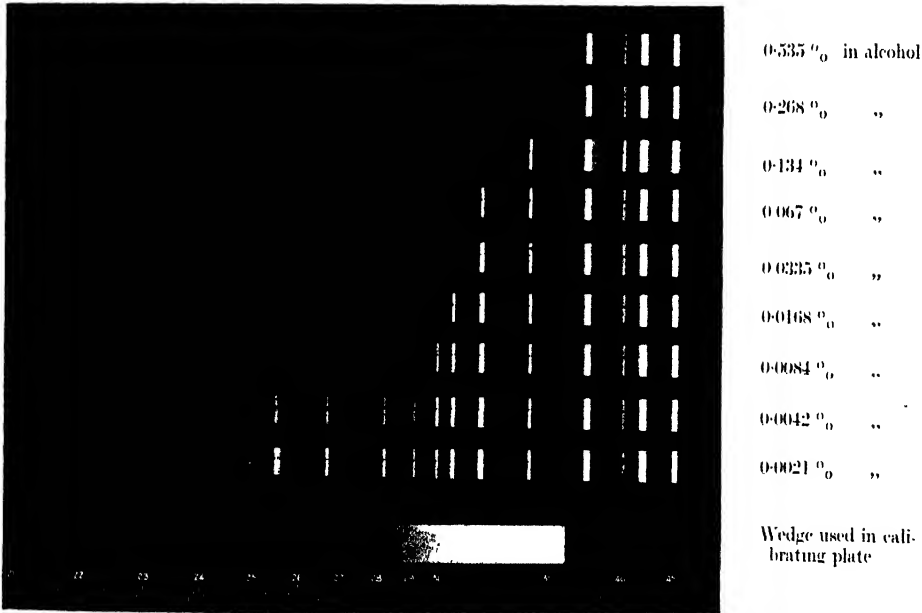


Fig. 2. Products of irradiation after removal of ergosterol.

In Figs. 1 and 2 it should be noted (a) that in Fig. 1 (ergosterol) absorption is still marked at 270 to 290 in a concentration of 0.0062 % although line 254 is clearly visible, whereas in Fig. 2 at concentration 0.0084 % the absorption between 270 and 290 is no greater than that at 254; (b) that in Fig. 2 at concentration 0.067 % the line 302 has entirely disappeared, although in Fig. 1 this line is still prominent even at 0.1 % concentration.



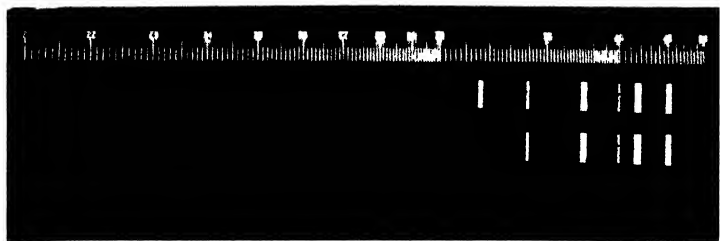


Fig. 3. Ergosterol 0.2 % in ether.



Fig. 4. Ergosterol 0.1 % in ether.

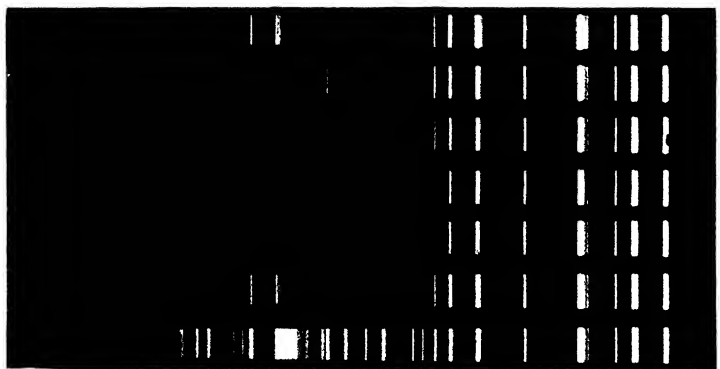


Fig. 5. Ergosterol 0.01 % in alcohol.

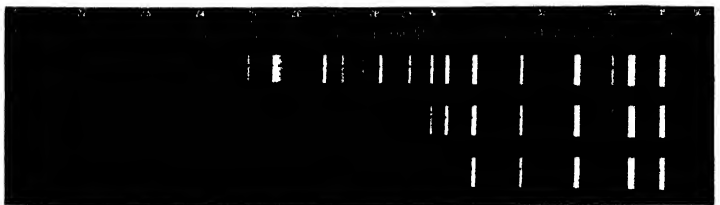


Fig. 6. Products of irradiation after removal of ergosterol



# CLIII. THE DESTRUCTIVE ACTION OF HEAT ON INSULIN SOLUTIONS.

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*(Received August 26th, 1928.)*

WE have endeavoured to determine the relationship between temperature and destruction of sterile watery buffered solutions of insulin (about 15 international units per mg.) and to find a mathematical expression for it. According to the law of mass action we might expect that the rate at which insulin is destroyed at a constant temperature at any moment should be proportional to its concentration as expressed by the well-known equation

$$k = \frac{1}{t} \cdot \log \frac{a}{a-x}$$

where  $k$  is the velocity constant,  $a$  the initial concentration, and  $x$  the amount destroyed at the time  $t$ . This expression can be used to evaluate  $k$ , the quantity  $(a - x)$  left at a given time  $t$  being estimated by biological standardisation.

Experiments were carried out at 50°, 60°, 80°, 100° and 117°.

The constant temperatures were obtained by using an electrical incubator (at 50°, 60° and 80°), boiling water or vapour from boiling water (100°) and vapour from boiling normal primary butyl alcohol (117°).

For the experiments at 50°, 60°, 80° and 100° the solution was placed in rubber-capped vials, whereas at 117° we used test-tubes (Jena Fiolax glass) sealed in the blow-pipe. They were placed at the constant temperatures and after the lapse of a certain period of time they were taken out and instantly cooled in water. The potency of each sample was compared with the potency of a sample of the same solution which had been left in the ice-chest, where no measurable destruction takes place within very long periods of time. The constants evaluated from the experiments are given in Table I.

In order to decide whether or not the equation employed holds good for the destruction process we have made experiments at 60° (one experiment), 100° (three experiments), and 117° (two experiments) in which samples (each in a separate vial) were taken out at various periods of time. By placing a thermometer in one of the vials used at 100° and a metallic alloy melting just below 117° in one of the tubes used at that temperature it was found that the time elapsing before the liquid in the tubes became heated to the surrounding temperature could have no appreciable influence on the result (i.e. the error



of the time of heating would not exceed 1 % even in the shortest experiment). The results of the experiments are given in Table I.

Table I.

Temp. ° C.	<i>t</i> (hours)	Initial $p_H^*$	$\frac{a-x}{a}$	<i>k</i>	<i>a</i> (units per cc.)	Brand†	Test†	No. in Fig. 2
50.1	623.0	4.5	0.66	0.000296	20	Leo	r.	1
50.1	623.0	3.5	0.60	0.000360	15	Leo	r.	2
60.0	192.0	4.0	0.52	0.00147	20	Leo	m.	3
60.0	360.0	4.0	0.39	0.00113	20	Leo	m.	4
60.0	696.0	4.0	0.116	0.00134	20	Leo	m.	—
60.0	1030.0	4.0	0.076	0.00109	20	Leo	m.	—
60.0	143.0	4.5	0.77	0.000797	—	Leo	r.	5
60.3	181.0	3.5	0.59	0.00128	—	Leo	r.	6
60.0	308.5	—	0.47	0.00107	—	Leo	r.	7
80.0	16.0	—	0.82	0.00537	—	B.D.H.	r.	—
79.7	16.2	3.5	0.52	0.0178	—	Leo	r.	8
81.0	20.0	—	0.36	0.0219	—	Leo	r.	9
100.0	1.0	4.0	0.74	0.131	20	Leo	m.	—
100.0	2.0	4.0	0.43	0.183	20	Leo	m.	—
100.0	3.08	4.0	0.425	0.121	20	Leo	m.	—
100.0	4.0	4.0	0.29	0.134	20	Leo	m.	—
100.0	6.0	4.0	0.35	0.076	20	Leo	m.	—
100.0	8.0	4.0	0.38	0.053	20	Leo	m.	—
100.0	2.5	4.0	0.475	0.129	20	Leo	m.	10
100.0	8.0	4.0	0.195	0.089	20	Leo	m.	—
100.0	1.73	4.0	0.50	0.174	40	Leo	m.	11
100.0	3.5	4.0	0.27	0.163	40	Leo	m.	—
100.0	5.26	4.0	0.228	0.122	40	Leo	m.	—
100.0	7.0	4.0	0.125	0.129	40	Leo	m.	—
100.0	9.3	4.0	0.074	0.121	40	Leo	m.	—
100.0	10.53	4.0	0.046	0.127	40	Leo	m.	—
100.0	2.0	—	0.50	0.150	—	B.D.H.	r.	12
100.0	2.0	4.0	0.57	0.124	—	Leo	r.	13
100.0	2.0	3.5	0.60	0.111	—	B.W.	r.	14
117.0	0.542	4.0	0.415	0.705	20	Leo	m.	15
117.0	1.04	4.0	0.355	0.432	20	Leo	m.	—
117.0	2.21	4.0	0.177	0.34	20	Leo	m.	—
117.0	2.73	4.0	0.135	0.319	20	Leo	m.	—
117.5	1.0	4.0	0.332	0.478	40	Leo	m.	16
117.5	2.05	4.0	0.18	0.362	40	Leo	m.	—
117.5	3.0	4.0	0.10	0.33	40	Leo	m.	—
117.5	4.0	4.0	0.048	0.33	40	Leo	m.	—
117.5	5.0	4.0	0.029	0.308	40	Leo	m.	—

\* At the time when the experiments were made the colorimetric method of  $p_H$  determination was used (bromophenol blue). When later on the hydrogen electrode was employed it turned out that a considerable correction had to be made. Thus in reality the values of  $p_H = 4$  in the table correspond to  $p_H = 3.2$  as measured by the hydrogen electrode.

† Abbreviations: B.D.H., British Drug Houses; B.W., Burroughs Wellcome; r., rabbit test; m., mouse test.

From the equation quoted above the logarithm of undestroyed insulin ( $a - x$ ) can be expressed as a linear function of heating time,  $t$ ,

$$\log(a - x) = -kt + \log a.$$

Since  $a$  is the initial concentration,  $\log a$  is constant, and accordingly the results obtained from the experiments at 60°, 100° and 117° should be represented by a straight line when plotting heating time against the logarithm of undestroyed insulin. In Fig. 1 this is illustrated in the case of one of the experiments (at 117.5°). Apparently a line connecting the points would in

some of the experiments have a tendency to bend upwards during its course, indicating that the destruction becomes relatively slower as the activity decreases, but in no cases except perhaps in one experiment at 100° can this be said with certainty. The velocity constant calculated from different points at the same temperature shows variations of 50–100 % (see Table I) probably for the most part owing to experimental errors, but perhaps also to some extent because the equation fails to express quite exactly the mechanism of the process.

However, the variations of the constant calculated for the same temperature are negligible when compared with the difference between the averages of the constants calculated for different temperatures, *e.g.* the constant at

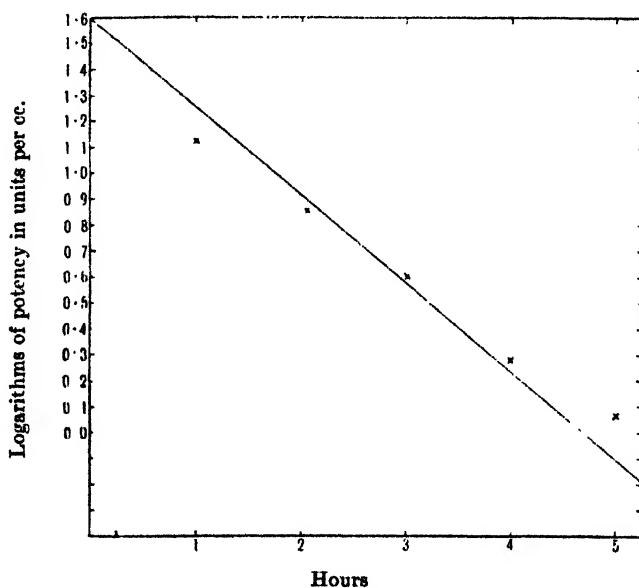


Fig. 1.

100° is a hundred times larger than the constant at 60°, and the constant at 117° is about three times as large as the constant at 100°.

In spite of the rather large dispersion of the constants for each temperature the wide range of temperatures employed appears sufficient as regards the determination of the exact relation of velocity constant to temperature.

Arrhenius's relation between absolute temperature  $T$  and the velocity coefficient  $k$  can be written in terms of a linear relation

$$\log_{10} k = -\frac{0.43 \cdot \mu}{2} \cdot \frac{1}{T} + \text{constant},$$

and its applicability can be tested by plotting  $\log k$  against  $1/T$ .

As seen in Fig. 2 the process follows closely the law of Arrhenius, at least within the limits of temperatures employed. Only constants evaluated from

nearly the same stage of destruction, namely half destruction, have been employed since this seems to be the most proper way of comparison.

In view of the wide divergence of temperatures examined it seems fairly probable that the relation found will be valid over an even wider range of temperatures. Assuming this, the time taken for the activity to fall 5, 10 and 50 % has been calculated for a series of lower temperatures, at which it is difficult to make experiments owing to the slow rate of destruction. The results are given in Table II.

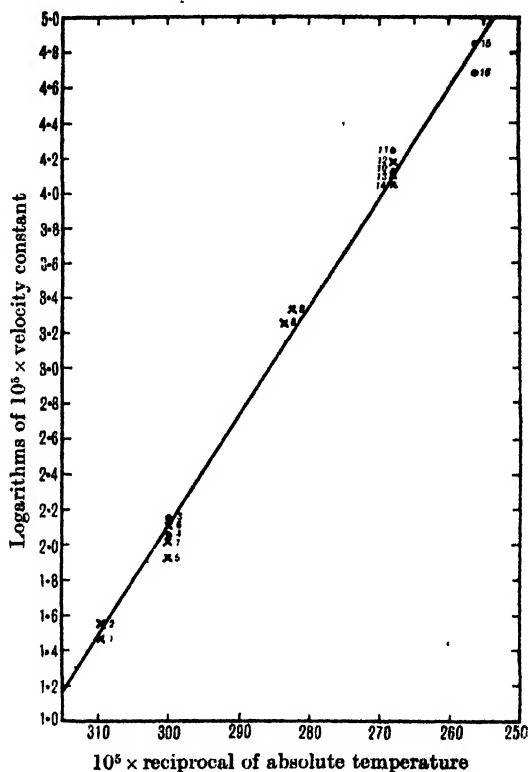


Fig. 2.

Table II.

	Time taken for the activity to fall		
	5 %	10 %	50 %
40°	$\frac{1}{2}$ month	1 month	$\frac{1}{2}$ year
30°	2 months	4 months	2 years
20°	9 "	1 $\frac{1}{2}$ years	10 "
10°	4 $\frac{1}{2}$ years	8 $\frac{1}{2}$ years	56 "
0°	29 "	58 "	377 "

Since the values given in Table II are extrapolated values, the value for 0° will be much less accurate than those for 30° and 40°. Judging from calculations

based upon displacement of the straight line in Fig. 2 into extreme and improbable situations the accuracy of the values for  $0^\circ$  is 50–100 %, and for  $30^\circ$  about 25 %.

The value of the constant  $\mu$  calculated from the straight line drawn in Fig. 2 is 28,300, which is a very high value as compared to the values met with in most chemical reactions. Generally  $\mu$  is between 10,000 and 17,000. High values of  $\mu$  are by some authors especially ascribed to monomolecular reactions. Whether the apparent change in the relative velocity during a single destruction experiment may be due to the formation of products inhibiting the destruction or to some other reason, must be the aim of future research. We ought to mention that there is invariably a precipitation of inactive material at this  $p_H$  during the heating process and that there is a shift of  $p_H$  during the destruction process in spite of the buffer. Applying the hydrogen electrode at  $18^\circ$  to a solution before and after 2 hours' heating at  $100^\circ$  we found a shift of  $p_H$  from 3.24 to 3.40. As standard electrode the Veibel quinhydrone electrode was used [Büllmann, 1925].

The influence of temperature on the buffer action of the solutions employed was tried by placing the same solution at  $18^\circ$  and at  $70^\circ$  (in a water-bath covered with oil) and applying a hydrogen electrode. As a standard electrode at  $70^\circ$  another hydrogen electrode was used in 0.01 *N* HCl + 0.09 *N* KCl which was supposed to have the same  $p_H$  at  $18^\circ$  and  $70^\circ$ . Owing to the high vapour pressure at  $70^\circ$  the experiment could not be of long duration. A constant potential was rapidly obtained corresponding to  $p_H = 3.31$ . The same solution showed  $p_H = 3.24$  at  $18^\circ$ . Applying to another solution the hydrogen electrode at  $18^\circ$ ,  $25^\circ$  and  $37^\circ$  (in water thermostats), using the Veibel quinhydrone electrode as a standard electrode and applying its temperature coefficient as quoted by Büllmann [1925], the shift of  $p_H$  was found to be 0.01. We conclude from these experiments that there is no large shift of  $p_H$  caused by temperature or taking place in the course of a destruction experiment. But as we know nothing exactly about the influence of  $p_H$  on the rate of destruction the shifts of  $p_H$  might to some extent be the cause of the dispersion of the constants.

For the purpose of determining the influence of shifts of  $p_H$  on the destruction rate a number of solutions were colorimetrically adjusted to different values of  $p_H$  and supplied with buffers, and the  $p_H$  values were checked by hydrogen electrode measurements. The solutions adjusted to  $p_H$  about 4 and 6.5 have in most cases been slightly opalescent.

The buffers added were such as have no temperature coefficient according to Walbum [1920], at least between  $18^\circ$  and  $70^\circ$ , namely the citrate-HCl mixtures and the phosphate mixtures of Sørensen [1909]. The influence of temperature on the buffer action of insulin itself is very slight according to the experiments quoted above (when the buffer added was also one that had no temperature coefficient between  $18^\circ$  and  $70^\circ$ ).

As seen in Table III the colorimetric method and the electrometric method do not agree when bromophenol blue and bromocresol green are used as

indicators. All the electrometrically obtained values given are calculated from potentials which had attained a constant value (*i.e.* constant within about half a millivolt or less in the course of a quarter of an hour). For the calculation of the values of  $p_H$  1-3 the Bjerrum [1911] extrapolation formula has been applied in order to eliminate the liquid junction potential, first using 3.5 *N* KCl and then using 1.75 *N* KCl to connect the two electrodes. In the other cases only 3.5 *N* KCl was used. For all the measurements one of two platinum electrodes was used which had been shown to produce the same potential when used in the same solution.

Table III.

Batch	$p_H$		Buffer (i)	$t$ (hours)	$a$	$a-x$	$k$	Temp. ° C.	
	Color.	Electr.							
A	1.2	(a)	1.10	1.0	0.75	18.7	11.9	0.261 (g)	100.8
B	1.2	(a)	1.17	1.0	1.0	20.0	11.0	0.26 (g)	100.2
B	1.2	(a)	1.17	1.0	1.225	20.0	9.9	0.249 (h)	100.6
C	2.0 $\pm 0.1$	(a)	1.73	0.25	1.975	20.0	11.1	0.130 (h)	100.1
D	2.0 $\pm 0.1$	(a)	1.79	0.25	1.975	20.0	11.5	0.122 (h)	100.1
E	1.9	(a)	1.78	0.25	4.0	20.0	5.1	0.149 (g)	100.4
A	1.9	(a)	1.76	0.50	2.0	13.3	8.1	0.108 (g)	100.0
C	2.45 $\pm 0.15$	(a)	2.42	0.25	1.975	20.0	10.9	0.134 (h)	100.1
D	2.45 $\pm 0.15$	(a)	2.34	0.25	1.975	20.0	11.8	0.116 (h)	100.1
E	2.8 $\pm 0.5$	(a), (b)	—	0.25	3.987	20.0	8.6	0.092 (g)	100.4
A	3.0	(a), (b)	2.88	0.57	2.0	11.0	6.1	0.128 (g)	100.0
C	4.2 $\pm 0.2$	(c)	3.63	0.25	1.975	20.0	10.8	0.135 (h)	100.1
D	4.2 $\pm 0.2$	(c)	3.65	0.25	1.975	20.0	9.8	0.157 (h)	100.1
E	4.0	(d)	3.58	0.25	3.975	20.0	7.2	0.112 (g)	100.4
C	6.5 $\pm 0.2$	(d)	6.75 (f)	0.25	0.975	20.0	7.4	0.432 (h)	100.6
C	6.5 $\pm 0.2$	(d)	6.75 (f)	0.025	0.975	20.0	10.3	0.289 (h)	100.6
B	7.45 $\pm 0.3$	(e)	7.43	0.80	0.0833	20.0	12.7	2.36 (h)	100.2

(a) thymol blue; (b) bromophenol blue; (c) bromocresol green; (d) bromophenol blue; (e) cresol red; (f) in reality this solution was not electrometrically checked: three other different solutions prepared in a similar way were colorimetrically adjusted to  $p_H = 6.5 \pm 0.2$  and showed  $p_H = 6.81$ , 6.75 and 6.70 with the hydrogen electrode; (g) sealed test-tubes; (h) rubber-capped vials; (i) buffer content expressed in proportion to buffer content of Sørensen's standards of the same  $p_H$ .

The solutions were submitted to a temperature of 100° for a period of time supposed to cause half destruction and then compared with a sample of the same solution which had been submitted to 100° for 1.5 minutes. Since this is the time taken for the vials to reach 100° the error of a too low constant in short experiments will be avoided provided that 1.5 minutes is subtracted from the apparent heating time when evaluating the constant.

The results of these experiments are given in Table III and Fig. 3. It is seen from the figure that the influence of the slight  $p_H$  variations in the destruction experiments is too small to account for the dispersion of the constants in either Fig. 2 or 3, when  $p_H$  is about 3. For the purpose of comparing the dispersion of the constants in Figs. 2 and 3 the logarithms of the constants have been chosen as ordinates also in Fig. 3, and the results from the experiments at 100° in Fig. 2 have been inserted at  $p_H$  3.2 in Fig. 3.

For the purpose of mutual control some of the estimations of insulin activity have been performed by the rabbit blood-sugar test as described by Marks [1926] and others by the mouse convulsion test as described by Krogh

and Hemmingsen [1926]. In Fig. 2 the points obtained from the mouse method (marked  $\bullet$ ) show good agreement with the points obtained by the rabbit method (marked  $\times$ ).

A closer study reveals that the dispersion of the constants for each temperature is larger than might be expected from the accuracy usually obtained with the methods of assay under consideration. This must be ascribed to unknown sources of error in the destruction experiments. It ought also to be mentioned that single determinations of partly destroyed samples show a tendency to larger dispersion than do undestroyed samples, perhaps because of different conditions of resorption in the animals.

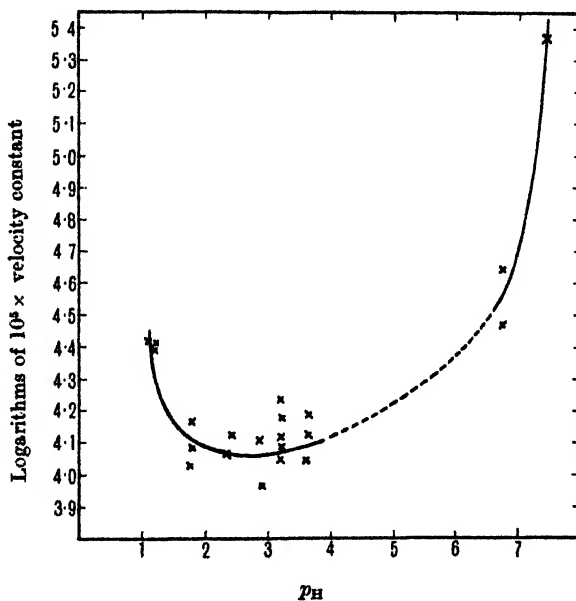


Fig. 3.

#### SUMMARY.

1. The destruction of insulin at a constant temperature is found to follow fairly well the law of monomolecular reactions, the rate being at any moment proportional to the concentration.

2. The relation of rate of destruction to temperature can be well expressed by the formula of Arrhenius. The critical thermal increment amounts to 28,300.

3. The rate of destruction is not appreciably altered by shifts of  $p_H$  when this is between 2 and 4.

We are greatly indebted to Prof. E. Biilmann and Mr A. Klit for the use of the apparatus for the electrometric determination of  $p_H$  at the Chemical Laboratory of the University of Copenhagen, and for the benefit of their special knowledge in this field of work.

## REFERENCES.

- Biilmann (1925). *L'Électrode à quinhydrone et ses applications* (Masson, Paris).  
Bjerrum (1911). *Z. Elektrochem.* **17**, 58, 389.  
Marks (1926). Health Organisation of League of Nations. Document C.H. 398.  
Krogh and Hemmingsen (1926). Health Organisation of League of Nations.  
Document C.H. 398, p. 40.  
Sørensen (1909). *Compt. Rend. Lab. Carlsberg*, **8**, i, 396.  
Walbum (1920). *Biochem. Z.* **107**, 219.

# CLIV. ON THE MEASUREMENT OF CHLORINE ION POTENTIALS IN THE PRESENCE OF PROTEINS.

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*(Received August 23rd, 1928.)*

CALOMEL and silver-silver chloride electrodes have been used extensively in the physical chemistry of the proteins for determining the concentration of chlorine ions in the presence of proteins [Bugarsky and Liebermann, 1898; Manabe and Matula, 1913; Oryng and Pauli, 1915; Pauli and Matula, 1917; Loeb, 1922; Northrop and Kunitz, 1924]. A few attempts have also been made to use these electrodes for chlorine determinations in biological fluids. Barkus [1924] reported some estimations of  $\text{Cl}'$  in gastric juice using  $\text{Ag-AgCl}$  electrodes; Neuhausen and Marshall [1922] used similar electrodes to find out whether all the chlorine in serum is free and diffusible; and Brown and Hill [1921] attempted to show the difference in chlorine concentration between oxygenated and reduced blood by the same means. On the other hand Warburg [1922] stated that such electrodes (concentration chains of the second order) in the presence of protein will not give potentials that conform with the Nernst equation, and that their use is therefore unsound; he argued this on purely theoretical grounds and brought no experimental evidence in support of it. In the following experiments an attempt was made to use the silver-silver chloride electrode for the determination of chlorine in human blood-plasma; it proved unsuccessful but, especially in view of Warburg's criticism, the observations seem worth recording.

## APPARATUS AND TECHNIQUE.

The following chain was used:

$\text{Ag-AgCl} \mid 0.02 \text{ molar KCl} \mid \text{“unknown”} \mid \text{AgCl-Ag}.$

The electrodes were made of pure silver wire, bent twice at their lower ends and covered over the bent parts with an electrolytically deposited layer of silver chloride; they were mounted on rubber corks fitted into glass electrode vessels with side tubes for making electrical contact with each other. In one vessel were placed about 4 cc. of a standard solution of pure 0.02  $M$   $\text{KCl}$ , in the other a similar quantity of the plasma or other solution under investigation; a direct liquid junction was effected by bringing the free ends of the side tubes



of the vessels into apposition, and slipping a short piece of rubber tubing over them. The potential difference between the two electrodes was then measured with a Cambridge and Paul direct-reading potentiometer, using a recently checked Weston cell; the result could be read to 0.0001 volt, which gave a deflection of 1 mm. on the galvanometer scale.

Before every experiment six electrodes were prepared by being cleaned and re-coated with fresh layers of silver and silver chloride. Individual variations between electrodes were then avoided by choosing two which gave no P.D. when immersed in the same KCl solution. These two were set up in the electrode vessels, which along with the standard cell were kept at a uniform temperature of  $20^\circ \pm 0.02^\circ$ , in an air thermostat of the kind described by Clark [1922]. Potentiometer readings were taken at varying intervals of time after the closure of the liquid junction.

#### OBSERVATIONS ON PURE KCl SOLUTIONS.

A preliminary series of observations was made on pure KCl solutions of known concentration, as a test of the reliability and accuracy of the apparatus and technique. The solutions were all approximately 0.1 *M*. The potentiometer readings sometimes showed fluctuations of a few tenths of a millivolt in the first 10 minutes after closure of the liquid junction, but after that in every case they remained steady to the nearest 0.0001 volt. They were observed at frequent intervals for periods varying from 25 to 140 minutes; breaking the junction and making a fresh one did not cause any change in the reading.

The logarithms of the concentrations of the solutions, and the E.M.F. obtained are given in Table I, and plotted in Fig. 1. The points lie on a straight line, as might be expected (over this narrow range of concentrations) from the equation for the E.M.F. of a concentration chain of this kind, viz.

$$E = \frac{2\eta RT}{nF} \ln \frac{c_1 \gamma_1}{c_2 \gamma_2},$$

where  $E$  = E.M.F. in volts;  $\eta$  = transference number of cation = 0.496 for K;  $R$  = gas constant = 8.316;  $T$  = absolute temperature;  $n$  = valency;  $F$  = Faraday's constant;  $c_1, c_2$  = concentrations of anion;  $\gamma_1, \gamma_2$  = activity coefficients of anion.

From this straight line the E.M.F. corresponding to a Cl<sup>-</sup> concentration of 0.1 mol. per 1000 g. water can be read. It is 0.0370 volt. If this be substituted for  $E$  in the above equation, then since the ratio  $c_1/c_2$  is known to be 0.10/0.02 and the values of the constants are known,  $\gamma_1/\gamma_2$  can be calculated and is found to be 0.876. This agrees with the results of Noyes and McInnes [1920] who obtained the values  $\gamma_1 = 0.745$  and  $\gamma_2 = 0.849$  (by interpolation), giving a ratio of 0.878.

The preliminary series of observations therefore showed that the apparatus and technique were adequate; the E.M.F.'s obtained with known KCl solutions were steady, reproducible (as shown by the straight line in Fig. 1) and in agreement with standard observations.

## OBSERVATIONS ON KCl SOLUTIONS CONTAINING PROTEIN.

The next step was to find out whether steady and reproducible E.M.F. could be obtained from solutions of known chlorine concentration, containing protein. For this purpose sheep or ox serum was dialysed in collodion tubes in a cool room against either 0.03 *M* sodium bicarbonate, or 0.03 *M* buffer solution of sodium acetate and acetic acid of  $p_H$  5.5, until the dialysate gave no precipitate with silver nitrate; in one or two cases the protein solution was also tested for chloride by ashing and extraction with dilute nitric acid and found to be chlorine-free. The final concentration of the protein solution was determined by micro-Kjeldahl estimation of the nitrogen, and known amounts of pure KCl were then added by weight. Four control solutions were also prepared by dissolving KCl in the bicarbonate or acetate solutions used in the dialysis.

Observations of E.M.F. were made on 20 solutions in all; their composition and the final readings obtained are shown in Table I (Nos. 11 to 30).

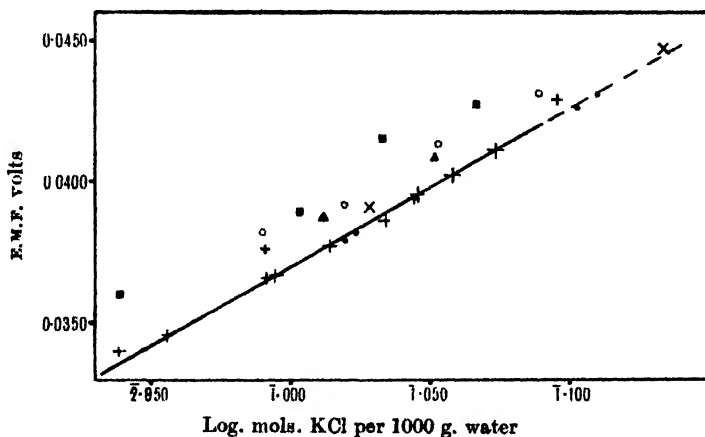


Fig. 1. The relation of the E.M.F. to the logarithm of the concentration of KCl (Table I).

- + : Solutions 1 to 9, containing nothing but KCl in water.
- : Solutions 11 to 14, containing acetate or bicarbonate
- ▲ : Solutions 15 to 17, containing bicarbonate and 2.7 % protein.
- : Solutions 27 to 30, containing bicarbonate and 8.5 % protein.
- ⊕ : Solutions 20 to 22, containing acetate and 3 % protein.
- x : Solutions 18, 19, containing acetate and 7.8 % protein.
- : Solutions 23 to 26, containing acetate and 10 % protein.

The E.M.F. of the solutions containing salts but no protein, Nos. 11 to 14, usually fell a few tenths of a millivolt in the first 10 or 15 minutes after closure of the liquid junction, and thereafter remained satisfactorily steady.

In a solution containing protein, however, the E.M.F. was by no means steady. It was found that a slight mechanical disturbance, such as tapping the upper end of the electrode dipping into the protein solution, would regularly cause a sharp rise in the E.M.F.—a millivolt or so—followed by a gradual fall.

This difficulty was overcome by keeping the electrode in a state of continual vibration. The gong of an electric bell was removed and its clapper replaced by a flagellum of iron wire about 8 inches long. The brass connecting piece carried by the top end of the electrode wire was covered with insulating tape, and the end of the flagellum brought up against it. When the bell current was turned on, the "whipping" of the brass piece by the flagellum kept the upper and the lower ends of the electrode wire in a state of continuous agitation, which had the additional advantage of stirring the liquid in the electrode vessel.

Table I.

No.	Concentration of protein %	Salts present (other than KCl) 0.03 M	Log. mols. KCl per 100 g. water	E.M.F. volts
1	—	—	2.9943	0.0367
2	—	—	2.9381	0.0340
3	—	—	1.0585	0.0402
4	—	—	2.9912	0.0366
5	—	—	2.9558	0.0346
6	—	—	1.0134	0.0377
7	—	—	1.0339	0.0386
8	—	—	1.0445	0.0395
9	—	—	1.0735	0.0410
11	—	Sodium acetate and acetic acid	1.1101	0.0431
12	—	" "	1.1030	0.0426
13	—	" "	1.0186	0.0379
14	—	Sodium bicarbonate	1.0227	0.0382
15	2.7	" "	1.0112	0.0387
16	2.7	" "	1.0514	0.0408
17	2.7	" "	1.0899	0.0431
18	7.8	Sodium acetate and acetic acid	1.1339	0.0447
19	7.8	" "	1.0281	0.0391
20	3.0	" "	1.0441	0.0404
21	3.0	" "	2.9904	0.0376
22	3.0	" "	1.0956	0.0429
23	10.0	" "	1.0526	0.0413
24	10.0	" "	1.0894	0.0431
25	10.0	" "	2.9896	0.0382
26	10.0	" "	1.0188	0.0392
27	8.5	Sodium bicarbonate	1.0029	0.0389
28	8.5	" "	2.9388	0.0360
29	8.5	" "	1.0663	0.0427
30	8.5	" "	1.0326	0.0415

When this device was applied to the electrode in the protein solution, the E.M.F. readings showed a gradual fall of 1 to 2 millivolts in the first hour or so, after which they remained practically steady. Sometimes there was a slight drift of about 0.0001 volt per hour.

The whipping device was therefore used in all the observations on solutions 11 to 30. It was also used in the case of No. 9 in the first series, where it was found to make no difference whatever to the steadiness or to the absolute value of the reading in the case of a pure KCl solution.

The reproducibility of the readings obtained on the solutions containing protein is best assessed by plotting the E.M.F. against the logarithms of the known concentrations of chlorine (in mols. per 1000 g. water). This is done in Fig. 1, where the straight line is that given by the preliminary observations. It will be seen that the points for the four solutions (Nos. 11 to 14) containing

no protein practically coincide with this line, showing that the electrode is uninfluenced by the sodium acetate or bicarbonate present. On the other hand, the points for the solutions containing protein (Nos. 15 to 30) all lie above the line, by anything up to 0.0023 volt. This means that the presence of the protein alters the relationship of the E.M.F. to the concentration of chlorine. The discrepancy is not very great, but it seems to be irregular, for the deviation of the points in Fig. 1 from the straight line bears no relation either to the concentration of protein present or to the hydrogen ion concentration (bicarbonate or acetate buffer). So long as this discrepancy is unexplained or cannot be allowed for, the silver-silver chloride electrode, with this technique, cannot be used for the accurate estimation of chlorides in the presence of protein.

## OBSERVATIONS ON OXALATED HUMAN PLASMA.

Haemoglobin-free plasma was obtained by centrifuging oxalated blood obtained from arm veins, and the potentials given by an electrode dipping into this plasma were observed with the technique already described. It was found impossible to get a satisfactorily steady reading. Nine attempts were made. Sometimes the reading showed an irregular drift; sometimes it showed an initial fall, a steady period, and then either a subsequent rise or a subsequent fall; in one case only it remained steady for a reasonable period (from the 97th to the 292nd minute). Moreover, if two sets of readings were made on different portions of the same specimen of plasma, at an interval of several hours or 1 day, they were found to be completely different. The unsteadiness of the readings, and this utter lack of reproducibility, are illustrated by the following figures:

(a) Fresh plasma		(b) Further quantity of same plasma 3 hours later: same electrode	
Time (minutes)	Reading (volts)	Time (minutes)	Reading (volts)
0	Liquid junction made	2	0.0501
3	0.0403	8	0.0525
10	0.0412	20	0.0530
24	0.0424	31	0.0515
37	0.0420	44	0.0500
50	0.0416	55	0.0482
63	0.0413	65	0.0469
99	0.0412	74	0.0462
136	0.0411	97	0.0452
154	0.0411	120	0.0447
		209	0.0440
		236	0.0438
		262	0.0436

When potentials were determined on successive days on the same plasma using a fresh electrode for each occasion an increase of 7 millivolts was observed on the second day. This would suggest that changes occur in the plasma with time which affect the activity of the chlorine ion. On the other hand, electrodes which had been immersed in plasma for 2 to 5½ hours gave, after washing, values of  $E$  in a pure solution of KCl which differed by 5 to 7 millivolts from those recorded before treatment with plasma. This is evidence that plasma has a definite "poisoning" effect on the electrode.

## DISCUSSION.

*The observations on pure KCl solutions.* It is interesting to note that the E.M.F. in the case of No. 9 solution, in which the electrode was kept vibrating, is in conformity with those obtained in the other solutions where the electrode was still; this throws doubt on Brown and Hill's [1921] conclusion that in such measurements as these the liquid must be kept still.

*The observations on KCl solutions containing protein* gave E.M.F.'s which are all higher than was expected. The solutions appear to contain a greater concentration of Cl' than is known to be present (or else the activity coefficient of the ion is raised). Possible explanations of this are as follows.

1. There may be a disparity in the concentrations of ions between the solution inside the protein micellae and the solution surrounding them. Since the proteins are on the alkaline side of their isoelectric point, the concentration of chlorine outside the micellae (and therefore affecting the electrode) might be expected to be greater than that inside, and therefore greater than the average chlorine concentration of the whole solution. If this were the explanation, however, the apparent increase in chlorine concentration as judged from the E.M.F. should bear some relation to the concentration of protein present and to the hydrogen ion concentration; no such relation appears in the readings that were obtained.

2. The protein molecules or ions may be hydrated. Again, if this were the explanation, the apparent increase in chlorine concentration should be related to the protein concentration present. Moreover, it has been shown by Corran and Lewis [1922], in the case of sucrose, which is known to be hydrated in solution, that the activity of chlorine in the presence of sucrose is the same as in its absence, the concentrations being calculated in the total water present (free water plus water of hydration).

3. The behaviour of the electrode may be modified by the presence of protein. Oryng and Pauli [1915], using calomel electrodes, found a lower E.M.F. with salt-free dialysed serum-protein than with distilled water; in the chain they used this meant that serum-protein after being shaken with calomel appeared to have a higher concentration of chlorine than water shaken with calomel. In the light of other experiments they attributed this to the formation and ionisation of a mercury-protein complex, and in subsequent work they introduced a correction for this effect, assuming it to be proportional to the concentration of protein present. Warburg [1922] criticises this. He lays down two conditions theoretically necessary for the determination of Cl' electro-metrically:

(a) that the Hg or Ag ion concentration be determined solely by the activity of the Cl'; this is not the case if metal-protein complexes be formed;

(b) that none of the other solutes be affected by the Hg or Ag ions.

He considers that in the presence of protein neither of these conditions is fulfilled, and the determination is impossible.

On the whole, the third of these suggested explanations seems to carry most weight. In the absence of further positive findings, the results that have been given provide an experimental justification for the criticism that Warburg made on purely theoretical grounds.

*The observations on oxalated plasma* show that in whole plasma there are more disturbing factors at work than simply the presence of protein, and the E.M.F. obtained are quite unreliable as a measure of the chlorine present. What constituent of plasma is responsible for the "poisoning" effect described was not determined, for in view of the unsatisfactory results obtained with the protein solutions it seems very unlikely that the electrometric method can be applied with success to the estimation of chlorine in plasma.

#### SUMMARY.

Chlorine ion potentials were measured by means of silver-silver chloride electrodes. The readings obtained in pure potassium chloride solutions were steady and reproducible. The readings obtained in potassium chloride solutions containing serum-protein were steady, but not accurately reproducible, and indicated a higher concentration of chlorine ions than was known to be present. The readings obtained in oxalated human plasma were not steady, and were not reproducible. Immersion in plasma "poisoned" the electrode. The technique as used is not applicable to the estimation of the chlorine ion in plasma.

#### REFERENCES.

- Barkus (1924). *Amer. J. Physiol.* **68**, 349.  
Brown and Hill (1921). *J. Physiol.* **54**; *Proc.* cix.  
Bugarsky and Liebermann (1898). *Pflüger's Arch.* **72**, 51.  
Clark (1922). The determination of hydrogen ions (Baltimore).  
Corran and Lewis (1922). *J. Amer. Chem. Soc.* **44**, 1673.  
Loeb (1922). Proteins and the theory of colloidal behaviour.  
Manabe and Matula (1913). *Biochem. Z.* **52**, 369.  
Neuhausen and Marshall (1922). *J. Biol. Chem.* **53**, 365.  
Northrop and Kunitz (1924). *J. Gen. Physiol.* **7**, 25.  
Noyes and McInnes (1920). *J. Amer. Chem. Soc.* **42**, 230.  
Oryng and Pauli (1915). *Biochem. Z.* **70**, 368.  
Pauli and Matula (1917). *Biochem. Z.* **80**, 187.  
Warburg (1922). *Biochem. J.* **16**, 153.

## CLV. THE DETERMINATION OF CARNOSINE.

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*(Received July 30th, 1928.)*

EARLY in the year 1921 Clifford published a method of estimating carnosine, which is based on the colour produced when carnosine reacts with diazobenzene-sulphonic acid. The resultant coloured solution is matched in a colorimeter against a standardised mixture of Congo red and methyl orange [Koessler and Hanke, 1919].

The method was extended to the estimation of chromogenic substances in muscle (mainly, no doubt, carnosine). The muscle, in this process, is minced, weighed, and extracted with water, the proteins in the extract precipitated by metaphosphoric acid, and the filtrate treated as if it were a carnosine solution. Based on this method a series of papers has been published [Clifford, 1921, 1, 2, 1922, 1, 2, 1923] with results which are consistent with each other, and which form a starting-point for the investigation of the functions of carnosine.

Unfortunately the other two workers who have concerned themselves with carnosine estimations do not agree with Clifford in some, and those the simplest and most striking, of her findings. G. Hunter has sorely criticised many details of the method [Hunter, 1921, 1922, 1924] and even suggests that the carnosine nitrate upon which Clifford's estimations are based was only 43 % pure. As this was supplied to her by Dr H. H. Dale as pure, and as the melting-point was correct, it is a suggestion not easily accepted. Mitsuda [1923] agrees with Hunter in saying that the carnosine content of cats' muscle varies.

Now this disagreement is intolerable, and it was resolved to re-examine some of the salient points of difference. The experimental work has been shared, so that one of us (V. H. M.) was able to hand over to the other (W. M. C.) clear, colourless solutions for estimation, the source, previous history, and carnosine content of which were quite unknown to the estimator.

### METHOD OF ESTIMATION.

#### (1) *On solutions of pure carnosine.*

The method follows that of Koessler and Hanke [1919]. To 10 cc. of 1.1 % sodium carbonate solution are added 2- $x$  cc. of distilled water, 4 cc. of the diazo-reagent (kept cool by ice in hot weather) and  $x$  cc. of the solution

to be estimated. The volume of the last naturally varies inversely as the strength of the solution. The mixed solutions are left standing at room temperature for 3–5 minutes to develop the full colour. Then the colour is estimated in a Duboscq colorimeter with Koessler and Hanke's C.R.–M.O. standard mixture as a comparison. No deviations from the Koessler and Hanke technique have been adopted except that the minute directions as regards time of mixing the reagents have not been found necessary, and the strength of the indicator chosen is such that a match at 30 mm. with the carnosine solution set at 20 mm. corresponds to 0.0001 g. of carnosine.

(2) *On tissues containing iminazole compounds.*

The tissue is minced, and from 1 to 6 g. are weighed accurately into a small Monax beaker. From 80 to 90 cc. of distilled water are added, and the whole brought slowly to the boil. 5 cc. of a 20 % solution of metaphosphoric acid are then added, and the mixture is allowed to stand (best overnight). The mixture is filtered and the precipitate washed. The filtrate is made up to 100 cc. Of this crystal clear fluid about 1 cc. is used for the estimation and the technique is exactly that of the preceding paragraph.

REINVESTIGATION OF THE METHOD.

(1) *On pure carnosine.*

(a) *Accuracy of the method.* As the original work was carried out in the difficult post-war period when dyes for the C.R.–M.O. indicator were hard to obtain, and as the original sample of carnosine nitrate, guaranteed pure as it was by Dr Dale, was rather small, it appeared to us necessary to re-standardise the old indicator (the stock solutions of which are still in use) with a new pure sample of carnosine.

Attempts to make in this laboratory, or to purchase on the market, samples of carnosine having proved abortive, this crucial test could not have been made if it had not been for the kindness of Dr Dudley, to whom we are indebted for half a gram of the pure base carnosine.

Solutions of known strength were made in distilled water from this sample and estimated, with the results shown in Table I. Carefully calibrated pipettes, measuring flasks and burettes were used.

Table I.

Solution	Calculated %	Found %	Difference g.
1	0.1460	0.146 (5)	+0.0005
2	0.0730	0.073	Nil
3	0.0487	0.050	+0.0063
4	0.0365	0.036 (6)	+0.0001
5	0.3595	0.359	–0.0005
6	0.2200	0.219	–0.0010
7	0.6190	0.617	–0.0020
8	0.3650	0.365	Nil



The figures speak for themselves. With one exception (Solution 3), the agreement is remarkably close, and in this case there is a note in the protocol (V. H. M.) that the emptying of the automatic pipette did not appear satisfactory. Neglecting this one solution, the agreement is little short of perfect. There seemed to be no reason for going any further with this part of the work, which indicates (i) that the original carnosine was not, as has been suggested, 43 % pure only, but chemically pure; (ii) that the stock solutions of dyes keep perfectly satisfactorily over a number of years; and (iii) that the method of estimation has a high order of accuracy.

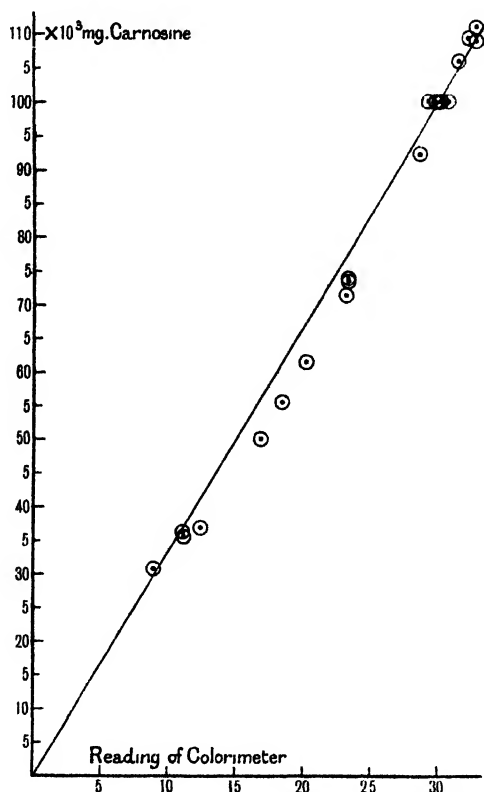


Fig. 1. Readings on the colorimeter plotted against carnosine content of solution.

The straight line represents the true linear relation.

(b) *Linear agreement between colour and amount of carnosine.* It has been stated [Clifford, 1921, 1] that the production of diazo-colour is not directly proportional to the amount of carnosine present in the solution to be estimated, whereas Hunter [1921] claims a complete proportionality. Mitsuda [1923], while agreeing with Clifford that the relation is not linear, states that the deviation from linearity is not so great as Clifford found.

In making the estimations given in Table I some 32 adjustments of the strengths of the solutions were made before the end-point was reached. Fortunately, accurate colorimeter readings were taken of the matches given before the correct dilutions were obtained, so that we have a large number of readings comparing colour produced with the known amounts of carnosine present. These are given in Fig. 1. It will be seen that there is a fair approximation to linearity. None the less, the best and most normally displaced from the mean are those when the reading of the C.R.-M.O. in the colorimeter is at 30 mm. and the unknown is set at 20 mm., the conditions under which estimations are usually made.

(2) *On muscle extracts.*

The chief interest of a method of estimating carnosine lies in its applicability to tissues. There are often large amounts in vertebrate muscle of substances which give the diazo-reaction. The iminazole compounds present are carnosine, purine compounds, histamine and possibly histidine; but no one doubts that it is carnosine which is responsible for the major part of the colour. Hunter [1922] estimates it at 95 % of the total, so that an accurate and consistent method of estimating the chromogenic substances in muscle is likely to throw some light upon the functions of carnosine.

Now one thing which appeared certain from Clifford's work [1921, 2; 1922, 2] on the distribution of carnosine in animal tissues, is that all the skeletal muscles of any animal of any one species have an almost constant amount of carnosine, but that each species has its own constant which may range from zero (*e.g.* cod, sole, finch) to 1 % (*e.g.* ox).

Both Hunter and Mitsuda maintain, on the other hand, that there is no constancy in the carnosine content of different animals of the same species. Estimates are given for the gastrocnemius of the cat which run from 0.007 % to 1.293 %, while Clifford's figures lie between 0.48 % and 0.51 %.

To eliminate the personal factor in the colour comparisons a large series of estimations of the chromogenic substances in ox and cat muscle has been made, using 13 different muscles or groups of muscle in the ox and 9 different muscles and 4 groups of muscle in the cat. In all these estimations except the last four, by which time it was considered proved that the personal factor did not count at all, the weighings and the extractions of the muscle were made by one of us (V. H. M.) and nothing but clear solutions handed over to the other (W. M. C.) for estimation.

Bullocks' muscle is a convenient tissue to investigate, but it must be freshly killed and not imported [Clifford, 1922, 1]. Various "cuts" were bought on various dates and the chromogenic substances found are expressed as carnosine in Table II. Each figure given is the mean of at least three parallel estimations agreeing within 1 %—indeed the greatest difference between highest and lowest estimate of any one sample is 8 parts in 970.

Table II. *Chromogenic substances in bullocks' muscle expressed as carnosine.*

"Cut"	Carnosine %
Rump steak	0.983
Shoulder steak	0.990
Fillet steak	0.981
Flank	0.981
Silver side	0.974
Shin	0.981
Skirt	0.975

It is obvious that there is great constancy in the amount. The figures show that the amount of carnosine is constant from animal to animal and for different groups of muscle. To get discrete muscles and not "cuts," five pairs and an odd one were dissected from a bullock's head. Their analyses are given in Table III.

Table III. *Chromogenic substances in muscles of bullock's head expressed as carnosine.*

Name of muscle	Carnosine %
Superior constrictor (?)	0.987, 0.991
Masseter	0.986
Buccinator	0.994, 0.991
Trapezius	0.991, 0.986
Pterygoideus externus	0.992, 0.990
" internus	0.990, 0.991

It thus appears from the results in Tables II and III that the carnosine content of the different skeletal muscles of the ox is approximately constant both from muscle to muscle and from animal to animal. The greatest variation is from 0.975 % to 0.994 %—at the most a little under 2 %.

Table IV. *Chromogenic substances in cats' muscles expressed as carnosine.*

No. of Exp.	Name of muscle	Carnosine %	Notes
1	Gastrocnemius	0.500, 0.496	
	Psoas	0.499, 0.496	
	Gluteus max. (right)	0.490	
	" (left)	0.492	
	Soleus	0.483	
	Tensor fasciae	0.485	
	Triceps brachialis	0.484, 0.483	
	Semi-membranosus	0.495, 0.498	
	Quadriceps	0.480, 0.482	
	Biceps femoris	0.498, 0.500	
2	—	0.517, 0.509, 0.515	Killed 10. iii. 28. In cold room till 12. iii. 28. Muscle in (4) smells of ether
3	—	0.511, 0.513, 0.514	
4	—	0.484, 0.477, 0.485	Killed 12. iii. 28. Sent that day. Smells slightly of ether
5	—	0.482, 0.487, 0.480	

It is, however, mainly with the cat that Hunter and Mitsuda claim to have found differences in carnosine content between muscle and muscle and cat and cat, though they state that the right gastrocnemius has the same amount as the left. A careful dissection of one cat yielded nine muscles in reasonable amounts for estimation, and we obtained, in addition, from the Medical Research Institute, through the kindness of Dr Dale, four carcasses of

cats which had been used for experiments there. From these, portions of muscle were taken, but no careful dissection into separate muscles was made. The results are given in Table IV.

It is hardly necessary to comment on the figures of Table IV. No case can be made from them for a wide variation of the carnosine content of skeletal muscle from muscle to muscle, or from animal to animal.

The difference between the highest and the lowest is but 8 % of the lowest, whereas Mitsuda's figures run (for the gastrocnemius) from 0.684 % to 1.293 % and Hunter's from 0.007 % to 0.433 %.

#### DISCUSSION.

The results detailed above, though going far to substantiate the adequacy and accuracy of Clifford's method for the estimation of carnosine, do little to clear up the confusion. This method has been used with great accuracy to determine the amount of carnosine in solutions of strength known to one person but not to the estimator. Further, the carnosine content of various solutions, colourless and giving no clue as to their content on inspection, has been shown to be proportional to the amount of tissue from which they came. Care was taken by the one who carried out the weighings that there should be great differences in the weight of tissue used, whereas the final solution handed over to the estimator was always 100 cc. The weight of tissue was varied 600 % without any effect on the final result. Consequently we consider that these figures make out a good case for the estimation of carnosine by Clifford's method, though we admit that we cannot understand or explain why other workers get such discordant results.

#### SUMMARY.

1. A reinvestigation of Clifford's method of estimation of carnosine shows that the original carnosine upon which the work was based was pure and that the indicators, once standardised, were satisfactory.
2. By this method the carnosine content of a solution of pure carnosine in water can be estimated with an error less than 1 %.
3. Using this method to estimate the chromogenic substances in samples of muscle, it has been shown that they vary in amount directly as the amount of muscle taken, no matter what skeletal muscle is used, or if it be from a different animal of the same species. In other words, for the skeletal muscle of a species the carnosine content is a constant. This constant varies from species to species though not from member to member of that species.
4. This work on ox and cat muscle confirms observations on the muscles of rats, rabbits, calves, sheep and lambs [Clifford, 1921, 2].

The expenses of this research were defrayed by a grant from the Medical Research Council.

## REFERENCES.

- Clifford (1921, 1). *Biochem. J.* **15**, 400.  
— (1921, 2). *Biochem. J.* **15**, 725.  
— (1922, 1). *Biochem. J.* **16**, 341.  
— (1922, 2). *Biochem. J.* **16**, 792.  
— (1923). *Biochem. J.* **17**, 549.  
Hunter (1921). *Biochem. J.* **15**, 689.  
— (1922). *Biochem. J.* **16**, 640.  
— (1924). *Biochem. J.* **18**, 408.  
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 497.  
Mitsuda (1923). *Biochem. J.* **17**, 630.

# CLVI. NEUTRAL SALT ADDITION COMPOUNDS OF *N*-METHYLATED GLYCINES: THEIR FORMULATION AND THAT OF THEIR HYDRATES.

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*(Received August 31st, 1928.)*

It is now a well-established fact that some of the naturally occurring  $\alpha$ -amino-acids have the property of forming addition compounds with neutral salts, such as the halides of the alkali and alkaline earth metals. It is probable that all exhibit this property to some extent in solution, but the isolation of crystalline additive products is only successful where the solubility of the addition product is less than that of the amino-acid or the inorganic salt, a condition of infrequent occurrence.

Glycine,  $\text{NH}_2\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$ , furnishes the best example of an extended series of addition compounds [Pfeiffer and Modelski, 1912, 1913; King and Palmer, 1920] and its simple derivatives sarcosine,  $\text{NHMe}\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$ , alanine,  $\text{NH}_2\cdot\text{CHMe}\cdot\text{CO}_2\text{H}$ , and betaine,  $\text{NMe}_3^+\cdot\text{CH}_2\cdot\text{CO}_2^-$ , have supplied a few further instances of this additive property [Pfeiffer, 1922].

The aim of the present investigation was primarily the use of *N*-dimethyl-aminoacetic acid,  $\text{NMe}_2\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$ , where the glycine type is retained, but where there are no longer any hydrogen atoms attached to nitrogen. This acid has given a particularly complete series of additive compounds with the chlorides, bromides and iodides of calcium, strontium, barium, lithium and sodium, but only one compound with a potassium halide. The composition of these complexes when compared with those, so far as they were known, of glycine, sarcosine and betaine revealed such striking analogies that the preparation of the missing additive compounds of sarcosine and betaine was also undertaken.

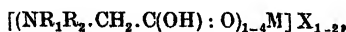
The complete series of additive compounds of glycine and its mono-, di-, and tri-methyl-derivatives is shown in the following table where G = glycine, S = sarcosine, D = dimethylglycine and B = betaine (anhydrous).

With one exception, all of these compounds belong to one of four types. They contain a metallic salt associated with 1, 2, 3 or 4 molecules of the amino-acid and for such there is only one consistent interpretation of their structure. The inorganic components are highly ionised salts and there is

Glycine	Sarcosine	Dimethylglycine	Betaine
G. CaCl <sub>2</sub> .3H <sub>2</sub> O <sup>a</sup>	S. CaCl <sub>2</sub> .4H <sub>2</sub> O <sup>b</sup>	D. CaCl <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>	—
G <sub>2</sub> .CaCl <sub>2</sub> .4H <sub>2</sub> O <sup>a, c, d</sup>	—	—	—
G <sub>3</sub> .CaCl <sub>2</sub> <sup>a</sup>	S <sub>3</sub> .CaCl <sub>2</sub> <sup>b</sup>	—	—
G <sub>2</sub> .CaBr <sub>2</sub> .4H <sub>2</sub> O <sup>a, d</sup>	—	D <sub>2</sub> .CaBr <sub>2</sub> .6H <sub>2</sub> O <sup>c</sup>	—
G <sub>2</sub> .CaBr <sub>2</sub> <sup>d</sup>	S <sub>2</sub> .CaBr <sub>2</sub> <sup>c</sup>	—	—
G <sub>2</sub> .CaI <sub>2</sub> .3H <sub>2</sub> O <sup>c, d</sup>	S <sub>2</sub> .CaI <sub>2</sub> .3H <sub>2</sub> O <sup>e</sup>	D. CaI <sub>2</sub> <sup>e</sup>	B <sub>5</sub> .(CaI <sub>2</sub> ) <sub>2</sub> .11H <sub>2</sub> O <sup>e</sup>
G <sub>4</sub> .CaI <sub>2</sub> <sup>d</sup>	—	—	—
G <sub>3</sub> .SrCl <sub>2</sub> .H <sub>2</sub> O <sup>c</sup>	S. SrCl <sub>2</sub> .4H <sub>2</sub> O <sup>b, e</sup>	D. SrCl <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>	B. SrCl <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>
G <sub>4</sub> .SrCl <sub>2</sub> .3H <sub>2</sub> O <sup>a</sup>	—	—	—
G <sub>2</sub> .SrBr <sub>2</sub> .3H <sub>2</sub> O <sup>a</sup>	S. SrBr <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>	D. SrBr <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>	B. SrBr <sub>2</sub> .5H <sub>2</sub> O <sup>e</sup>
G <sub>4</sub> .SrI <sub>2</sub> <sup>d</sup>	S <sub>3</sub> .SrI <sub>2</sub> .2H <sub>2</sub> O <sup>d</sup>	D <sub>2</sub> .SrI <sub>2</sub> .3H <sub>2</sub> O <sup>c</sup>	B <sub>2</sub> .SrI <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>
—	S. SrI <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>	—	—
G <sub>3</sub> .BaCl <sub>2</sub> .H <sub>2</sub> O <sup>a, c</sup>	S. BaCl <sub>2</sub> .4H <sub>2</sub> O <sup>b</sup>	D. BaCl <sub>2</sub> .4H <sub>2</sub> O <sup>c</sup>	B. BaCl <sub>2</sub> .4H <sub>2</sub> O <sup>a</sup>
—	S. BaBr <sub>2</sub> .4H <sub>2</sub> O <sup>b</sup>	D. BaBr <sub>2</sub> .4H <sub>2</sub> O <sup>c</sup>	B. BaBr <sub>2</sub> .4H <sub>2</sub> O <sup>a</sup>
G <sub>2</sub> .BaBr <sub>2</sub> .H <sub>2</sub> O <sup>a</sup>	—	—	B <sub>2</sub> .BaBr <sub>2</sub> .6H <sub>2</sub> O <sup>b</sup>
G <sub>4</sub> .BaI <sub>2</sub> <sup>d</sup>	S <sub>2</sub> .BaI <sub>2</sub> .2H <sub>2</sub> O <sup>e</sup>	D <sub>2</sub> .BaI <sub>2</sub> .4H <sub>2</sub> O <sup>c</sup>	B. BaI <sub>2</sub> .4H <sub>2</sub> O <sup>e</sup>
G. LiCl. H <sub>2</sub> O <sup>a, c</sup>	S. LiCl. H <sub>2</sub> O <sup>d, e</sup>	D. LiCl. 2H <sub>2</sub> O <sup>c</sup>	—
G <sub>2</sub> .LiCl. H <sub>2</sub> O <sup>a</sup>	—	—	—
G. LiBr. H <sub>2</sub> O <sup>a, c</sup>	S. LiBr. H <sub>2</sub> O <sup>d, e</sup>	D. LiBr. H <sub>2</sub> O <sup>c</sup>	B. LiBr. 2½H <sub>2</sub> O <sup>e</sup>
G <sub>2</sub> .LiBr. H <sub>2</sub> O <sup>a, c</sup>	—	D <sub>2</sub> .LiBr <sup>e</sup>	—
G. LiI. H <sub>2</sub> O <sup>d</sup>	S. LiI. 1½H <sub>2</sub> O <sup>d, e</sup>	D. LiI. 6H <sub>2</sub> O <sup>c</sup>	B <sub>2</sub> .LiI. H <sub>2</sub> O <sup>e</sup>
—	S. NaCl. H <sub>2</sub> O <sup>d</sup>	D. NaCl. 2H <sub>2</sub> O <sup>c</sup>	B. NaCl. 1½H <sub>2</sub> O <sup>e</sup>
G. NaBr. 1½H <sub>2</sub> O <sup>d</sup>	S. NaBr. H <sub>2</sub> O <sup>d</sup>	D. NaBr. H <sub>2</sub> O <sup>c</sup>	—
G <sub>2</sub> .NaBr. H <sub>2</sub> O <sup>b</sup>	—	D <sub>2</sub> .NaBr. 2H <sub>2</sub> O <sup>e</sup>	B <sub>2</sub> .NaBr. 1½H <sub>2</sub> O <sup>e</sup>
G <sub>2</sub> .NaI. H <sub>2</sub> O <sup>c, d</sup>	S. NaI. H <sub>2</sub> O <sup>b, d</sup>	D. NaI. 3H <sub>2</sub> O <sup>c</sup>	B. NaI. 3H <sub>2</sub> O <sup>e</sup>
—	S <sub>3</sub> .KCl. 5H <sub>2</sub> O <sup>e</sup>	—	—
—	S <sub>3</sub> .KBr. 4H <sub>2</sub> O <sup>b, e</sup>	—	B. KBr. 2H <sub>2</sub> O <sup>a</sup>
G <sub>4</sub> .KI <sub>3</sub> <sup>d</sup>	S <sub>3</sub> .KI <sub>3</sub> <sup>d</sup>	D. KI. 1½H <sub>2</sub> O <sup>c</sup>	B. KI. 2H <sub>2</sub> O <sup>a</sup>
—	S <sub>3</sub> .KI. 4H <sub>2</sub> O <sup>b</sup>	—	B <sub>2</sub> .KI. 2H <sub>2</sub> O <sup>a</sup>

<sup>a</sup> Pfeiffer and Modelski [1912].<sup>b</sup> Pfeiffer and Wittka [1915].<sup>c</sup> King and Paimer [1920].<sup>d</sup> Pfeiffer, Klossmann and Angern [1924].<sup>e</sup> Anslow and King (this paper).

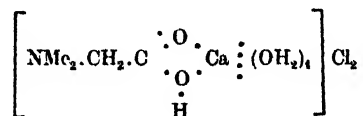
little doubt that they remain so in these complexes and are held by electrovalencies. The metallic cations are free to co-ordinate with the carboxyl oxygen atoms of the amino-acid molecules, two of the four electrons of the carbonyl oxygen atom entering the outer shell of the metallic cation in the manner suggested for the neutral salt addition compounds of alkaline earth glutamates and aspartates by Anslow and King [1927]. The compounds with sarcosine or glycine, for instance, where all four types are observed will have the general structure



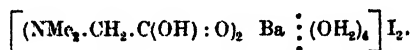
where M is the metallic cation co-ordinated through carbonyl oxygen to 1, 2, 3 or 4 amino-acid molecules and X is the halogen ion, the number, 1 or 2, depending on the electro-valency of M. On no other formulation, such for example as the combination of the halogen ion with the ammonium form of the amino-acid, does it seem possible to interpret such complexes as triglycine calcium bromide prepared by Pfeiffer, Klossmann and Angern [1924] or its sarcosine analogue, trisarcosine calcium bromide prepared by us.

So far, the formulation of the amino-acid molecules in their relation to the metallic atoms has been discussed to the exclusion of the water molecules.

The electronic arrangement of the oxygen atom in water is, however, similar to that of the carbonyl oxygen atom and the composition of the addition compounds formed between dimethylglycine and lithium bromide,  $D. LiBr. H_2O$  and  $D_2. LiBr$ , and between sarcosine and the potassium halides,  $S_3. KCl. 5H_2O$ ,  $S_4. KBr. 4H_2O$  and  $S_4. KI. 4H_2O$ , even suggests inter-equivalence in these cases of amino-acid and water molecules. If, therefore, the view be accepted that these additive compounds are held together by co-valencies, then in a series of about eighty compounds such as are enumerated here, one might by a consideration of the number of water molecules expect to find examples where the known maximum co-ordination number of the metallic element is exhibited. Such indeed is the case. Consider, for example, the additive compounds of dimethylglycine. The majority of the alkaline earth halide addition compounds are hydrated and most of them may be formulated as having a co-ordination number of 6. The commonest type is  $D. MX_2. 4H_2O$  and this falls into the scheme if each oxygen atom of the carboxyl group occupies one position in the co-ordination complex. Thus, dimethylglycine calcium chloride tetrahydrate would be written



whilst the type exemplified by di-dimethylglycine barium iodide tetrahydrate would be written



The exceptional hexahydration observed with di-dimethylglycine calcium bromide hexahydrate suggests either a co-ordination number of 8 for calcium as postulated by Pfeiffer in  $[\text{Ca}(\text{NH}_3)_8]\text{Cl}_2$  or attachment of the extra water molecules to the tertiary nitrogen atoms, one to each.

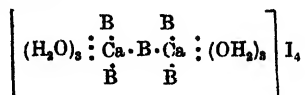
In a similar way the hydrates of the alkali halide addition compounds of dimethylglycine, with the exception of that with lithium iodide, may be formulated on the basis of a co-ordination number of 2 or 4 for the alkali metal. The same principles apply to the majority of the compounds of glycine, sarcosine and betaine.

One or two cases call for further comment. The exceptional series of compounds formed between sarcosine and the potassium halides mentioned above are most simply interpreted on a co-ordination number of 8 for potassium, the interchange of water and amino-acid molecules, and the odd number of amino-acid and water molecules in  $S_3. KCl. 5H_2O$ , being difficult to account for in any other way.

If the above views are correct, they ought to account for the most remarkable compound of this series yet observed. Betaine and calcium iodide form a well-authenticated addition compound containing eleven molecules of



water,  $B_2 \cdot (CaI_2)_2 \cdot 11H_2O$ . It crystallises in the cubic system and is optically isotropic. This is formulated as



where B is the hydrated form of betaine and calcium has the usual co-ordination number of 6. The use of betaine as a bridge between the two calcium atoms involves the use of both oxygen atoms of the carboxyl group, each occupying one co-ordination position of a calcium atom, and thus strengthens the formulation proposed above for the commonest type of compound observed between dimethylglycine and the alkaline earth halides,  $D.MX_2 \cdot 4H_2O$ .

### EXPERIMENTAL.

#### *Addition compounds of N-methylaminoacetic acid (sarcosine).*

*Tri-sarcosine calcium bromide.* Sarcosine hydrobromide (4.25 g.) was boiled with excess of calcium hydroxide; after treatment with carbon dioxide the solution was boiled, filtered and concentrated. On keeping, large, well-formed, rectangular plates were obtained (1.0 g.). These were filtered off rapidly, transferred to porous plate and well pressed to remove all adhering mother-liquor. (This method was used for all the addition compounds.)

0.2086 g.; 0.1717 g. AgBr. Br = 35.0 %.

0.1902 g.;  $NH_3$  = 14.48 cc. N/10 acid. N = 8.5 %.

Calc. for  $(C_2H_5O_2N)_3 \cdot CaBr_2$ : Br = 34.3 %; N = 9.0 %.

*Di-sarcosine calcium iodide trihydrate.* Square plates (1.9 g.) were obtained in the usual way from sarcosine hydriodide (4.3 g.) and excess calcium hydroxide. (Found: I, 48.1; N, 6.7 %; whence I : N = 1 : 1.26.) Recrystallisation gave elongated rectangular plates (0.5 g.) of the pure compound.

0.1720 g.; 0.1540 g. AgI. I = 48.4 %.

0.1740 g.;  $NH_3$  = 6.45 cc. N/10 acid. N = 5.2 %.

Calc. for  $(C_2H_5O_2N)_2 \cdot CaI_2 \cdot 3H_2O$ : I = 48.2 %; N = 5.3 %.

*Di-sarcosine barium iodide dihydrate.* Prepared in a similar way to the previous compound, this *dihydrate* crystallised in needles (0.9 g.).

0.2090 g.; 0.1607 g. AgI. I = 41.6 %.

0.2070 g.;  $NH_3$  = 6.53 cc. N/10 acid. N = 4.4 %.

Calc. for  $(C_2H_5O_2N)_2 \cdot BaI_2 \cdot 2H_2O$ : I = 41.9 %; N = 4.6 %.

*Sarcosine strontium chloride tetrahydrate.* This compound, previously prepared by Pfeiffer and Wittka [1915] from equimolecular solution, was obtained in needles from a solution of sarcosine and strontium chloride in the ratio 2 : 1. (Found: Cl, 21.7; N, 4.4 %. Theory: Cl, 22.2; N, 4.4 %.)

*Sarcosine strontium bromide tetrahydrate.* Needles (1.4 g.) were obtained in the usual manner.

0.2006 g. dried at 120° lost 0.0336 g.  $H_2O$  = 16.8 %.

0.2006 g.; 0.1810 g. AgBr. Br = 38.4 %.

0.2039 g.;  $NH_3$  = 5.36 cc. N/10 acid. N = 3.7 %.

Calc. for  $C_2H_5O_2N \cdot SrBr_2 \cdot 4H_2O$ ;  $H_2O$  = 17.6 %; Br = 39.1 %; N = 3.4 %.

*Sarcosine strontium iodide tetrahydrate.* Concentration of a solution prepared in the usual way gave needles (1.2 g.) which were impure. (Found: I, 48.2; N, 3.3 %; whence I : N = 1 : 0.62.) When, however, an equimolecular solution was concentrated needles (1.3 g.) of the pure compound were obtained.

0.2022 g.; 0.1872 g. AgI. I = 50.0 %.

0.2082 g.;  $\text{NH}_3$  = 4.10 cc. *N*/10 acid. N = 2.8 %.

Calc. for  $\text{C}_3\text{H}_7\text{O}_2\text{N} \cdot \text{SrI}_2 \cdot 4\text{H}_2\text{O}$ ; I = 50.5 %; N = 2.8 %.

*Tri-sarcosine potassium chloride pentahydrate.* Sarcosine hydrochloride (4.2 g.) was dissolved in 33.3 cc. *N* potassium hydroxide and the solution concentrated. After the removal of potassium chloride (1.1 g.), well-formed hexagonal plates were deposited (0.4 g.).

0.2080 g.; 0.0661 g. AgCl. Cl = 7.9 %.

0.1885 g.;  $\text{NH}_3$  = 13.04 cc. *N*/10 acid. N = 9.7 %.

Calc. for  $(\text{C}_3\text{H}_7\text{O}_2\text{N})_3 \cdot \text{KCl} \cdot 5\text{H}_2\text{O}$ ; Cl = 8.2 %; N = 9.7 %.

The existence of the tetra-sarcosine potassium bromide tetrahydrate of Pfeiffer and Wittka [1915] and of sarcosine lithium chloride monohydrate, sarcosine lithium bromide monohydrate and sarcosine lithium iodide sesquihydrate of Pfeiffer, Klossmann and Angern [1924] was confirmed.

#### *Salts of N-dimethylaminoacetic acid.*

*N-Dimethylaminoacetic acid hydrochloride.* This compound, described by Johnson [1906] and Friedmann [1908], was prepared by slowly dropping an aqueous solution of monochloroacetic acid (8.25 g. in 8.2 cc.) into a solution of dimethylamine (100 cc., 16.5 % aq.) cooled to 0°, followed by four hours' heating in a pressure bottle at 55°. An excess of baryta was added on cooling, and the liberated dimethylamine removed by concentration *in vacuo*. After exact precipitation of the barium by sulphuric acid and removal of the barium sulphate, concentration to a small volume (at 50°), followed by the addition of an equal volume of alcohol, gave a mass of transparent tablets, melting at 189–191°. The total yield was 8.75 g. (Found: Cl = 25.6 %. Theory: 25.2 %.)

The free acid was prepared by adding to a solution of the hydrochloride a slight excess of silver carbonate; after removal of the excess of silver with hydrogen sulphide, concentration gave very hygroscopic tablets which were dried *in vacuo* over sulphuric acid. This acid is readily soluble in dry methyl alcohol, but crystallises well in sheaves of needles on careful addition of acetone. M.P. 176–178° (Johnson gives M.P. 157–160°). (Found: N = 13.3 %. Theory: 13.6 %.)

The *chloroaurate* is dimorphous and very readily soluble in water. The less stable form separates as a microcrystalline pale yellow powder on concentration and in contact with the solution soon changes into deep orange rhombs. M.P. 96–98°.

0.1131 g. dried at 90° lost 0.0039 g.  $\text{H}_2\text{O}$  = 3.4 %.

0.1399 g.; 0.0687 g. Au. Au = 42.0 %.

Calc. for  $\text{C}_3\text{H}_7\text{O}_2\text{N} \cdot \text{HAuCl}_4 \cdot \text{H}_2\text{O}$ ;  $\text{H}_2\text{O}$  = 3.9 %; Au = 42.8 %.

*N*-Dimethylaminoacetic acid hydrobromide. Prepared by concentrating a solution of the amino-acid containing excess hydrobromic acid, this salt was obtained in anhydrous, elongated hexagonal plates which are slightly hygroscopic; it is less soluble than the hydrochloride. M.P. 158–160°.

0.2013 g.; 0.2053 g. AgBr. Br = 43.4 %.

Calc. for  $C_4H_9O_2N \cdot HBr$ ; Br = 43.4 %.

*N*-Dimethylaminoacetic acid hydriodides. The monohydriodide, prepared in a similar way to the hydrobromide, crystallises in hexagonal plates which are anhydrous and melt at 149°. Solutions become coloured on heating.

0.1964 g.; 0.1981 g. AgI. I = 54.5 %.

Calc. for  $C_4H_9O_2N \cdot HI$ ; I = 54.9 %.

The semi-hydriodide, prepared by using less hydriodic acid, concentrating to a small volume and adding alcohol until turbid, crystallised on standing overnight in tufts of broad needles. The recrystallised compound (a monohydrate) melts at 157°, but is decomposed if heated to 120° for half an hour.

0.2017 g.; 0.1326 g. AgI. I = 35.5 %.

0.2040 g.;  $NH_3$  = 11.94 cc. N/10 acid. N = 8.2 %.

Calc. for  $(C_4H_9O_2N)_2 \cdot HI \cdot H_2O$ ; I = 36.1 %; N = 8.0 %.

Attempts to prepare the semi-hydrochloride and semi-hydrobromide were unsuccessful—the less soluble normal salts contaminating the products obtained in each case, even after recrystallisation.

#### *Addition compounds of N-dimethylaminoacetic acid.*

*N*-Dimethylaminoacetic acid calcium chloride tetrahydrate. *N*-Dimethylaminoacetic acid hydrochloride (4.8 g.) dissolved in a small volume of water was boiled with excess of calcium hydroxide. Carbon dioxide was passed to remove the excess, and the solution was boiled to decompose carbamates, filtered and concentrated. After standing several days the syrupy solution deposited prismatic rods (1.25 g.).

0.2103 g. dried at 120° lost 0.0502 g.  $H_2O$  = 23.9 %.

0.2103 g.; 0.2050 g. AgCl. Cl = 24.1 %.

0.1951 g.;  $NH_3$  = 7.03 cc. N/10 acid. N = 5.0 %.

Calc. for  $C_4H_9O_2N \cdot CaCl_2 \cdot 4H_2O$ ;  $H_2O$  = 25.1 %; Cl = 24.8 %; N = 4.9 %.

*Di*-(*N*-dimethylaminoacetic acid) calcium bromide hexahydrate. *N*-Dimethylaminoacetic acid hydrobromide (6.1 g.) was boiled with excess calcium hydroxide in a similar way to the previous compound; 0.75 g. of long rods were obtained. This hexahydrate on drying loses  $4H_2O$  at 120° and a further  $\frac{1}{2}H_2O$  at 160°.

0.1945 g. dried at 120° lost 0.0271 g.  $H_2O$  = 13.9 %.

0.1500 g. dried at 160° lost 0.0239 g.  $H_2O$  = 15.9 %.

0.1945 g.; 0.1445 g. AgBr. Br = 31.6 %.

0.1993 g.;  $NH_3$  = 7.43 cc. N/10 acid. N = 5.2 %.

Calc. for  $(C_4H_9O_2N)_2 \cdot CaBr_2 \cdot 6H_2O$ ;  $4\frac{1}{2}H_2O$  = 14.0 %;  $4\frac{1}{2}H_2O$  = 15.8 %; Br = 31.1 %; N = 5.4 %.

A second less pure crop was obtained crystallising in needles.

*N*-Dimethylaminoacetic acid calcium iodide. *N*-Dimethylaminoacetic acid hydriodide (5.8 g.) was boiled with excess calcium hydroxide, and treated in the usual way; the solution deposited long needles (0.7 g.).

0.1937 g.; 0.1692 g. AgI. I = 47.2 %.

0.2022 g.;  $\text{NH}_3 = 7.40$  cc. *N*/10 acid. N = 5.1 %.

Calc. for  $\text{C}_4\text{H}_{10}\text{O}_2\text{N} \cdot \text{CaI}_2$ ; I = 47.0 %; N = 5.2 %.

*N*-Dimethylaminoacetic acid barium chloride tetrahydrate. Prepared under the foregoing conditions, long needles (0.9 g.) were obtained.

0.2003 g. dried at 120° lost 0.0391 g.  $\text{H}_2\text{O} = 19.3$  %.

0.2003 g.; 0.1464 g. AgCl. Cl = 18.1 %.

0.2057 g.;  $\text{NH}_3 = 5.78$  cc. *N*/10 acid. N = 3.9 %.

Calc. for  $\text{C}_4\text{H}_{10}\text{O}_2\text{N} \cdot \text{BaCl}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 18.8$  %; Cl = 18.5 %; N = 3.7 %.

A further crop was obtained on concentration.

*N*-Dimethylaminoacetic acid barium bromide tetrahydrate. Long needles (2.0 g.) were obtained under similar conditions.

0.2079 g. dried at 120° lost 0.0315 g.  $\text{H}_2\text{O} = 15.2$  %.

0.2079 g.; 0.1634 g. AgBr. Br = 33.5 %.

0.2017 g.;  $\text{NH}_3 = 4.61$  cc. *N*/10 acid. N = 3.2 %.

Calc. for  $\text{C}_4\text{H}_{10}\text{O}_2\text{N} \cdot \text{BaBr}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 15.3$  %; Br = 33.8 %; N = 3.0 %.

*Di*-(*N*-dimethylaminoacetic acid) barium iodide tetrahydrate. This compound was obtained in needles (0.6 g.) after recrystallisation of a slightly impure crop (1.6 g.) obtained in the usual way.

0.1943 g. dried at 120° lost 0.0186 g.  $\text{H}_2\text{O} = 9.6$  %.

0.1943 g.; 0.1338 g. AgI. I = 37.2 %.

0.1969 g.;  $\text{NH}_3 = 5.32$  cc. *N*/10 acid. N = 3.8 %.

Calc. for  $(\text{C}_4\text{H}_9\text{O}_2\text{N})_2 \cdot \text{BaI}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 10.8$  %; I = 37.9 %; N = 4.2 %.

*N*-Dimethylaminoacetic acid strontium chloride tetrahydrate. On concentration of an aqueous solution prepared in the usual way, this compound crystallised as a network of needles (2.6 g.).

0.1943 g. dried at 120° lost 0.0425 g.  $\text{H}_2\text{O} = 21.9$  %.

0.1943 g.; 0.1606 g. AgCl. Cl = 20.5 %.

0.2023 g.;  $\text{NH}_3 = 6.18$  cc. *N*/10 acid. N = 4.3 %.

Calc. for  $\text{C}_4\text{H}_{10}\text{O}_2\text{N} \cdot \text{SrCl}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 21.6$  %; Cl = 21.2 %; N = 4.2 %.

The mother-liquors gave a second crop on concentration.

*N*-Dimethylaminoacetic acid strontium bromide tetrahydrate. Under the foregoing conditions, long rods (2.9 g.) were obtained.

0.2035 g. dried at 120° lost 0.0335 g.  $\text{H}_2\text{O} = 16.5$  %.

0.2035 g.; 0.1784 g. AgBr. Br = 37.3 %.

0.1951 g.;  $\text{NH}_3 = 5.24$  cc. *N*/10 acid. N = 3.8 %.

Calc. for  $\text{C}_4\text{H}_{10}\text{O}_2\text{N} \cdot \text{SrBr}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 17.0$  %; Br = 37.8 %; N = 3.3 %.

A further crop was obtained on concentration.

*Di*-(*N*-dimethylaminoacetic acid) strontium iodide trihydrate. On concentration of a solution obtained in the usual way, a first crop (0.8 g.) was obtained slightly impure. (Found:  $\text{H}_2\text{O}$ , 9.2; I, 37.6; N, 4.8 %; whence I : N = 1 : 1.16.)

The second crop gave more correct figures:

0.1977 g. dried at 120° lost 0.0179 g.  $\text{H}_2\text{O} = 9.1$  %.

0.1977 g.; 0.1518 g. AgI. I = 41.5 %.

0.1792 g.;  $\text{NH}_3 = 6.07$  cc. *N*/10 acid. N = 4.7 %.

Calc. for  $(\text{C}_4\text{H}_9\text{O}_2\text{N})_2 \cdot \text{SrI}_2 \cdot 3\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 9.0$  %; I = 42.2 %; N = 4.7 %.

*N*-Dimethylaminoacetic acid lithium chloride dihydrate. *N*-Dimethylaminoacetic acid hydrochloride (4.8 g.) was boiled with lithium carbonate (1 mol.); concentration gave bold tablets (3.3 g.).

0.1973 g. dried at 120° lost 0.0388 g.  $H_2O = 19.7\%$ .

0.1973 g.; 0.1547 g. AgCl.  $Cl = 19.7\%$ .

0.2002 g.;  $NH_3 = 11.12$  cc.  $N/10$  acid.  $N = 7.8\%$ .

Calc. for  $C_4H_9O_2N \cdot LiCl \cdot 2H_2O$ ;  $H_2O = 19.9\%$ ;  $Cl = 19.5\%$ ;  $N = 7.7\%$ .

*N*-Dimethylaminoacetic acid lithium bromide monohydrate and di-(*N*-dimethylaminoacetic acid) lithium bromide. When an equimolecular solution of *N*-dimethylaminoacetic acid and lithium bromide was concentrated, long needles (1.3 g.) were obtained, which corresponded approximately to the compound  $C_4H_9O_2N \cdot LiBr \cdot 1\frac{1}{2}H_2O$  (Found:  $H_2O$ , 11.5; Br, 35.2; N, 6.7%; whence Br : N = 0.92 : 1); whilst on further concentration a crop of tablets (0.25 g.) of quite distinct appearance separated, approximating to the formula  $(C_4H_9O_2N)_2 \cdot LiBr$ . (Found: Br, 28.6; N, 9.1%; whence Br : N = 1.1 : 2.)

To isolate each of these two compounds in a pure condition, separate solutions containing lithium bromide and *N*-dimethylaminoacetic acid in the proportion of 2 : 1 and 1 : 2 were concentrated. The solution containing excess lithium bromide deposited rectangular plates (2.0 g.) which were deliquescent.

0.2078 g. dried at 120° lost 0.0212 g.  $H_2O = 10.2\%$ .

0.2078 g.; 0.1846 g. AgBr.  $Br = 37.8\%$ .

0.1970 g.;  $NH_3 = 9.79$  cc.  $N/10$  acid.  $N = 7.0\%$ .

Calc. for  $C_4H_9O_2N \cdot LiBr \cdot H_2O$ ;  $H_2O = 8.7\%$ ;  $Br = 38.4\%$ ;  $N = 6.7\%$ .

The second solution, containing two molecular proportions of *N*-dimethylaminoacetic acid, deposited hexagonal tablets (0.3 g.).

0.1212 g.; 0.0794 g. AgBr.  $Br = 27.9\%$ .

0.1457 g.;  $NH_3 = 9.80$  cc.  $N/10$  acid.  $N = 9.4\%$ .

Calc. for  $(C_4H_9O_2N)_2 \cdot LiBr$ ;  $Br = 27.3\%$ ;  $N = 9.5\%$ .

*N*-Dimethylaminoacetic acid lithium iodide hexahydrate. An equimolecular solution of lithium iodide and *N*-dimethylaminoacetic acid on concentration either on the water-bath or at room temperature, deposited mixed crystals. (Found in different preparations = I : N = 1 : 1.2.)

On this basis a third solution containing lithium iodide and *N*-dimethylaminoacetic acid in the proportion of 2 : 1 was concentrated at room temperature and gave rods (1.0 g.).

0.2112 g.; 0.1411 g. AgI.  $I = 36.1\%$ .

0.2080 g.;  $NH_3 = 6.02$  cc.  $N/10$  acid.  $N = 4.1\%$ .

Calc. for  $C_4H_9O_2N \cdot LiI \cdot 6H_2O$ ;  $I = 36.8\%$ ;  $N = 4.1\%$ .

Di-(*N*-dimethylaminoacetic acid) sodium chloride dihydrate. After several unsuccessful attempts with 1 : 1 solutions, this compound was obtained crystallising in irregular octahedra from a solution containing two molecules of *N*-dimethylaminoacetic acid to one of sodium chloride by evaporation in a desiccator over sulphuric acid. It gradually decomposes when dried at 120°.

0.1981 g.; 0.0995 g. AgCl.  $Cl = 12.4\%$ .

0.1946 g.;  $NH_3 = 12.67$  cc.  $N/10$  acid.  $N = 9.1\%$ .

Calc. for  $(C_4H_9O_2N)_2 \cdot NaCl \cdot 2H_2O$ ;  $Cl = 11.8\%$ ;  $N = 9.3\%$ .

*N*-Dimethylaminoacetic acid sodium bromide monohydrate and di(*N*-dimethylaminoacetic acid) sodium bromide sesquihydrate. Concentration of an equimolecular solution gave a mixture of long needles and hexagonal tablets; this mixture was re-dissolved and diluted, and on cooling tablets alone were obtained. (Found: Br, 30.2; N, 6.7%; whence Br : N = 1 : 1.27.) On one recrystallisation the plates obtained gave Br, 25.8; N, 7.9%; whence Br : N = 1 : 1.75, and after a further recrystallisation the pure sesquihydrate crystallising in rectangular tablets, which decompose on heating to 130°, was obtained.

0.1713 g.; 0.0969 g. AgBr. Br = 24.1%.

0.1904 g.; NH<sub>3</sub> = 11.07 cc. N/10 acid. N = 8.1%.

Calc. for (C<sub>4</sub>H<sub>9</sub>O<sub>2</sub>N)<sub>2</sub>.NaBr.1½H<sub>2</sub>O; Br = 23.8%; N = 8.3%.

For the isolation of the pure 1 : 1 compound indicated by the first analysis, a solution containing two molecules of sodium bromide to one of *N*-dimethylaminoacetic acid was concentrated; after removal of a small amount of sodium bromide, the required compound separated as a monohydrate in hexagonal tablets which decompose slightly on drying at 120°.

0.2108 g.; 0.1780 g. AgBr. Br = 35.9%.

0.1958 g.; NH<sub>3</sub> = 8.52 cc. N/10 acid. N = 6.1%.

Calc. for C<sub>4</sub>H<sub>9</sub>O<sub>2</sub>N.NaBr.H<sub>2</sub>O; Br = 35.7%; N = 6.3%.

*N*-Dimethylaminoacetic acid sodium iodide trihydrate. This compound crystallises in rectangular plates.

0.2095 g.; 0.1582 g. AgI. I = 40.8%.

0.2063 g.; NH<sub>3</sub> = 6.48 cc. N/10 acid. N = 4.4%.

Calc. for C<sub>4</sub>H<sub>9</sub>O<sub>2</sub>N.NaI.3H<sub>2</sub>O; I = 41.3%; N = 4.6%.

*N*-Dimethylaminoacetic acid potassium iodide sesquihydrate. The first crop obtained (rectangular plates) was impure. (Found: I, 38.8; N, 5.4%; whence I : N = 1 : 1.26); but a second crop of rectangular plates proved to be the pure compound.

0.1894 g.; 0.1504 g. AgI. I = 42.9%.

0.2070 g.; NH<sub>3</sub> = 7.03 cc. N/10 acid. N = 4.8%.

Calc. for C<sub>4</sub>H<sub>9</sub>O<sub>2</sub>N.KI.1½H<sub>2</sub>O; I = 42.9%; N = 4.7%.

Attempts to prepare addition compounds of potassium chloride or bromide were unsuccessful, the halide being recovered practically pure in each case.

#### *Addition compounds of N-trimethylaminoacetic acid (betaine).*

*Penta-betaine di-calcium iodide undecahydrate.* On concentration of a solution containing two molecules of betaine to one of calcium iodide, large, glistening, square plates were deposited which were optically isotropic. (Found: N, 5.2; I, 37.0%; whence N : I = 1 : 0.79.) Recrystallisation gave similar plates. (Found: N, 5.2; I, 37.6%; whence N : I = 1 : 0.8.)

When an equimolecular solution was concentrated, large, square plates were again obtained—with a similar ratio. (Found: N, 5.1; I, 37.5%; whence N : I = 1 : 0.8), and on recrystallisation the same compound was obtained.

0.1843 g. dried at 95° lost 0.0169 g.  $\text{H}_2\text{O} = 9.2\%$ .

0.1566 g.; 0.1081 g. AgI.  $\text{I} = 37.3\%$ .

0.1835 g.;  $\text{NH}_3 = 6.91$  cc.  $N/10$  acid.  $N = 5.3\%$ .

0.1843 g.;  $\text{C}_2\text{O}_4\text{Ca} = 5.48$  cc.  $N/10$   $\text{KMnO}_4$ .  $\text{Ca} = 6.0\%$ .

Calc. for  $(\text{C}_5\text{H}_{11}\text{O}_2\text{N})_5 \cdot (\text{CaI}_2)_2 \cdot 11\text{H}_2\text{O}$ ;  $7\text{H}_2\text{O} = 9.2\%$ ;  $\text{I} = 37.0\%$ ;  $N = 5.1\%$ ;  $\text{Ca} = 5.8\%$ .

Attempts to prepare compounds of calcium chloride or calcium bromide were unsuccessful, only syrupy solutions being obtained.

*Betaine barium iodide tetrahydrate.* Concentration of a solution containing two molecules of betaine to one of barium iodide gave woolly needles (0.3 g.) which were impure. (Found: N, 4.8; I, 32.2%; whence  $N : I = 1 : 0.75$ .) An equimolecular solution deposited a pure compound in rods (5.5 g.).

0.1959 g.; 0.1606 g. AgI.  $\text{I} = 44.3\%$ .

0.2080 g.;  $\text{NH}_3 = 3.36$  cc.  $N/10$  acid.  $N = 2.3\%$ .

Calc. for  $\text{C}_5\text{H}_{11}\text{O}_2\text{N} \cdot \text{BaI}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{I} = 43.7\%$ ;  $N = 2.4\%$ .

*Betaine strontium chloride tetrahydrate.* A solution of the components deposited long, silky needles (1.1 g.) on concentration.

0.1931 g. dried at 120° lost 0.0412 g.  $\text{H}_2\text{O} = 21.3\%$ .

0.1931 g.; 0.1558 g. AgCl.  $\text{Cl} = 20.0\%$ .

0.2100 g.;  $\text{NH}_3 = 6.11$  cc.  $N/10$  acid.  $N = 4.1\%$ .

Calc. for  $\text{C}_5\text{H}_{11}\text{O}_2\text{N} \cdot \text{SrCl}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 20.7\%$ ;  $\text{Cl} = 20.4\%$ ;  $N = 4.0\%$ .

*Betaine strontium bromide pentahydrate.* Concentration of a solution prepared in the usual way gave needles (1.0 g.) which were impure. (Found: N, 4.0; Br, 32.8%; whence  $N : \text{Br} = 1 : 1.4$ .) The mother-liquor on concentration, however, gave a crop of needles (0.3 g.) which were analytically pure.

0.0984 g. dried at 120° lost 0.0199 g.  $\text{H}_2\text{O} = 20.2\%$ .

0.0984 g.; 0.0824 g. AgBr.  $\text{Br} = 35.6\%$ .

0.0908 g.;  $\text{NH}_3 = 1.99$  cc.  $N/10$  acid.  $N = 3.1\%$ .

Calc. for  $\text{C}_5\text{H}_{11}\text{O}_2\text{N} \cdot \text{SrBr}_2 \cdot 5\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 19.8\%$ ;  $\text{Br} = 35.2\%$ ;  $N = 3.1\%$ .

*Di-betaine strontium iodide tetrahydrate.* This compound was obtained in the usual way, crystallising in short needles (0.3 g.).

0.1390 g.; 1015 g. AgI.  $\text{I} = 39.4\%$ .

0.0722 g.;  $\text{NH}_3 = 1.99$  cc.  $N/10$  acid.  $N = 3.9\%$ .

Calc. for  $(\text{C}_5\text{H}_{11}\text{O}_2\text{N})_2 \cdot \text{SrI}_2 \cdot 4\text{H}_2\text{O}$ ;  $\text{I} = 39.2\%$ ;  $N = 4.3\%$ .

*Betaine lithium bromide hydrate.* On concentration of the solution, prepared in the usual manner, rods (2.4 g.) were deposited: they were impure. (Found: N, 5.6; Br, 24.6%; whence  $N : \text{Br} = 1 : 0.77$ .) Recrystallisation gave long needles (0.5 g.) which were pure and contained  $2\frac{1}{2}$  molecules of water.

0.1910 g. dried at 120° lost 0.0348 g.  $\text{H}_2\text{O} = 18.2\%$ .

0.1910 g.; 0.1431 g. AgBr.  $\text{Br} = 31.9\%$ .

0.1918 g.;  $\text{NH}_3 = 7.52$  cc.  $N/10$  acid.  $N = 5.5\%$ .

Calc. for  $\text{C}_5\text{H}_{11}\text{O}_2\text{N} \cdot \text{LiBr} \cdot 2\frac{1}{2}\text{H}_2\text{O}$ ;  $\text{H}_2\text{O} = 18.1\%$ ;  $\text{Br} = 32.1\%$ ;  $N = 5.6\%$ .

*Di-betaine lithium iodide monohydrate.* Tufts of needles (1.0 g.) were obtained on concentration of an equimolecular solution.

0.1966 g.; 0.1198 g. AgI.  $\text{I} = 32.9\%$ .

0.2094 g.;  $\text{NH}_3 = 10.28$  cc.  $N/10$  acid.  $N = 6.9\%$ .

Calc. for  $(\text{C}_5\text{H}_{11}\text{O}_2\text{N})_2 \cdot \text{LiI} \cdot \text{H}_2\text{O}$ ;  $\text{I} = 32.9\%$ ;  $N = 7.2\%$ .

*Lithium chloride.* When an equimolecular solution of lithium chloride and betaine was concentrated to a syrup, a mixture of needles and small hexagonal

plates was slowly deposited. But when two solutions, one with the components in the ratio 2 : 1 and the other in the ratio 1 : 2, were concentrated, no crystalline substances could be isolated.

*Betaine sodium chloride sesquihydrate.* On concentration of an equimolecular solution a mixture was obtained. A second solution, however, containing twice the amount of betaine, when concentrated, gave long needles alone.

0.1918 g. dried at 120° lost 0.0294 g.  $H_2O = 15.3\%$ .

0.1918 g.; 0.1375 g. AgCl.  $Cl = 17.7\%$ .

0.2040 g.;  $NH_3 = 10.23$  cc.  $N/10$  acid.  $N = 7.0\%$ .

Calc. for  $C_5H_{11}O_2N \cdot NaCl \cdot 1\frac{1}{2}H_2O$ ;  $H_2O = 13.3\%$ ;  $Cl = 17.5\%$ ;  $N = 6.9\%$ .

*Di-betaine sodium bromide sesquihydrate.* The first crop obtained when an equimolecular solution was concentrated consisted of rods (1.1 g.) which were impure. (Found: N, 4.6; Br, 35.6%; whence  $N : Br = 1 : 1.36$ .) Concentration of the mother-liquor gave a second crop of glistening, rectangular leaflets (0.3 g.) which analysed correctly.

0.1078 g. dried at 120° lost 0.0093 g.  $H_2O = 8.6\%$ .

0.1078 g.; 0.0554 g. AgBr.  $Br = 21.9\%$ .

0.1359 g.;  $NH_3 = 7.23$  cc.  $N/10$  acid.  $N = 7.5\%$ .

Calc. for  $(C_5H_{11}O_2N)_2 \cdot NaBr \cdot 1\frac{1}{2}H_2O$ ;  $H_2O = 7.4\%$ ;  $Br = 21.9\%$ ;  $N = 7.7\%$ .

*Betaine sodium iodide trihydrate.* Rectangular plates (0.4 g.) were deposited on concentrating an equimolecular solution. They were slightly impure. (Found: N, 3.9; I, 38.1%; whence  $N : I = 1 : 1.08$ .) The second crop, also rectangular plates, proved to be pure.

0.1985 g.; 0.1449 g. AgI.  $I = 39.4\%$ .

0.2001 g.;  $NH_3 = 5.89$  cc.  $N/10$  acid.  $N = 4.1\%$ .

Calc. for  $C_5H_{11}O_2N \cdot NaI \cdot 3H_2O$ ;  $I = 39.2\%$ ;  $N = 4.4\%$ .

*Betaine ammonium iodide dihydrate.* An equimolecular solution deposited rods on concentration. (Found: N, 9.1; I, 45.1%; whence  $N : I = 1 : 0.54$ .)

The rods were recrystallised, and after removing a small quantity of leaflets, rods were again deposited.

0.2007 g. dried at 120° lost 0.0129 g.  $H_2O = 6.4\%$ .

0.2007 g.; 0.1575 g. AgI.  $I = 42.4\%$ .

0.2024 g.;  $NH_3 = 13.24$  cc.  $N/10$  acid.  $N = 9.2\%$ .

Calc. for  $C_5H_{11}O_2N \cdot NH_4I \cdot 2H_2O$ ;  $H_2O = 6.0\%$ ;  $I = 42.6\%$ ;  $N = 9.4\%$ .

From solutions of betaine and ammonium chloride, ammonium bromide and potassium chloride, no addition compounds could be obtained.

#### REFERENCES.

- Anslow and King (1927). *Biochem. J.* **21**, 1168.  
 Friedmann (1908). *Beitr. chem. Physiol. Path.* **11**, 195.  
 Johnson (1906). *Proc. Roy. Soc. Lond.* **78**, 82.  
 King and Palmer (1920). *Biochem. J.* **14**, 574.  
 Pfeiffer (1922). *Organische Molekülverbindungen* (Stuttgart).  
 Pfeiffer, Klossmann and Angern (1924). *Z. physiol. Chem.* **133**, 22.  
 Pfeiffer and Modelski (1912). *Z. physiol. Chem.* **81**, 329.  
 ——— (1913). *Z. physiol. Chem.* **85**, 1.  
 Pfeiffer and Wittka (1915). *Ber. deutsch. chem. Ges.* **48**, 1289.



## CLVII. THE SECOND PROTEIN (LIVETIN) OF EGG-YOLK.

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If the usual method is adopted for the preparation of lecitho-vitellin from hen's egg-yolk, namely, dilution of the egg-yolk with an equal volume of 10 % sodium chloride solution, thorough extraction of the mixture with ether, followed by dilution of the resulting clear aqueous layer with 15 to 20 volumes of water, it will be found that whilst the lecitho-vitellin is almost quantitatively precipitated, there is still an appreciable amount of protein-nitrogen in the filtrate [Gross, 1899, quoted by Plimmer]. Plimmer [1908] by acidifying the filtrate with acetic acid and boiling to coagulate the protein, obtained several grams of this material, which he called "livetin." He determined the nitrogen partition by the Hausmann method, and found that the figures agreed fairly closely with those obtained for vitellin, but differed from those obtained for ovalbumin. Since this work, little seems to have been done on this second protein of egg-yolk. Apart from the careful analyses of Plimmer just mentioned, we are not aware of any information as to its composition, or even as to the class of protein to which it belongs. Moreover Plimmer, although he brought evidence to show that his product was not ovalbumin, did not demonstrate clearly that the proteins of the white did not contribute to his coagulum. He put forward the suggestion that the product he separated might be closely related to vitellin—in fact, vitellin minus the phosphorus-containing fraction of the molecule.

During the course of the preparation of some pure ovovitellin we have taken the opportunity of examining our residues for this little studied substance, and are now able to present some more definite data as to its nature and properties.

### *Preparation of livetin.*

We find that it is difficult, if not impossible with some eggs, to free the yolk quantitatively from adherent white by washing it simply in running water as did Plimmer. To be quite sure that the yolk is clean, we find it necessary to wash it in three or four changes of 0.9 % sodium chloride (to dissolve the globulin satisfactorily), and also to remove the chalazae with scissors. The yolk is further cleaned by allowing it to roll slowly down a strip of dry

calico sheeting, returning it, if necessary, to be washed again in the salt solution. It is finally dried, as far as possible, on the sheeting. In this way the white can be completely removed. Lecitho-vitellin is prepared from the clean yolks in the usual way<sup>1</sup>, leaving invariably a considerable quantity of non-vitellin protein-nitrogen in the final filtrate. By half saturation with ammonium sulphate almost the whole of this protein may be precipitated. The filtrate from this precipitate gives only a very slight opalescence with trichloroacetic acid, thus demonstrating the absence of appreciable amounts of albumin. The precipitate is taken up with water, the concentration of ammonium sulphate brought up to 25 %, and the slight precipitate filtered off. To the clear filtrate sufficient saturated ammonium sulphate solution is added to bring the concentration of the salt to half saturation, the precipitated livetin filtered off, redissolved, and again precipitated. Solution and precipitation are repeated once more, the precipitated protein is then dissolved in the minimal quantity of water and the lipins are removed by alcohol-ether extraction at  $-15^{\circ}$  [Hewitt, 1927]. The extracted protein is freed from alcohol and ether by Hewitt's method, only a small fraction undergoing denaturation during the process. The dried, extracted protein, containing ammonium sulphate, is taken up in water, filtered, reprecipitated with ammonium sulphate, dissolved in the minimal quantity of water, and the solution, adjusted to  $p_H$  5.0, is dialysed in closed collodion sacs at  $3^{\circ}$  against repeated changes of distilled water until at least two days after the dialysate has ceased to give the faintest opalescence with barium chloride.

Although there are now only traces of salts in the sacs, the livetin is still in solution. Its behaviour is therefore that of a pseudoglobulin. In a few cases the dialysed solutions are opalescent, and may be clarified by filtration through paper pulp. On adding the clear, dialysed solution to 10 volumes of distilled water, an opalescence is produced, which disappears on the addition of dilute sodium chloride or ammonium sulphate solution. On continuing the dialysis for some weeks, a portion of the protein is precipitated.

#### EXAMINATION OF LIVETIN.

##### *Qualitative and precipitation tests.*

A 0.6 % solution of livetin gives a precipitate on boiling alone, or on boiling with very dilute acetic acid, a precipitate in the cold with three volumes of alcohol or acetone, with trichloroacetic, picric, tungstic and sulphosalicylic acids, with the salts of the heavy metals, and with colloidal ferric hydroxide. It is completely precipitated by half saturation with ammonium sulphate, by saturation with magnesium sulphate or sodium chloride (after 24 hours'

<sup>1</sup> A short dialysis of the lecitho-vitellin-NaCl solution against distilled water before precipitation of the phosphoprotein with water considerably reduces the amount of dilution necessary for its complete separation, and hence diminishes the bulk of the filtrate from which the livetin has to be precipitated.

standing in the case of the last two salts). It gives the biuret, xanthoproteic and Millon's reactions, a strong glyoxylic reaction, but a very weak, almost negative, Molisch reaction. With a 4 % solution of the protein, a positive sulphur reaction is given with sodium hydroxide and lead acetate. After hydrolysis with trypsin it gives a strong Pauly's reaction, and a definite but weak Totani's "secondary reaction" for histidine.

#### *Quantitative analysis.*

1. *Nitrogen, phosphorus and sulphur.* Livetin, prepared by adding to its solution in dilute ammonium sulphate, at the stage before alcohol-ether extraction, three volumes of alcohol and heating to boiling, gave, after washing the coagulated protein with water and alcohol, and drying at 100°, ash = 1 %; N = 15.12 %; P = 0.12 %. (Plimmer found N varying between 14.8 % and 15.2 %, and P values between 0.1 % and 0.65 % in five different specimens of livetin.)

A purer specimen after alcohol-ether extraction gave ash = 0.6 %; N = 15.35 %; P = 0.067 %, *i.e.* a much lower content of phosphorus. On our two purest specimens of livetin the N : P ratio was 1 g. N : 3.13 mg. P, and 1 g. N : 3.15 mg. P, respectively. This phosphorus is not extractable by alcohol-ether, either in the cold or on boiling. It is rather less than one-half of the amount of P per gram of N present in extracted egg-albumin [Hewitt, 1927]. Assuming that there is only one atom of P in each molecule of protein, the minimum molecular weight of livetin works out at about 64,000.

The total sulphur content of livetin is 1.80 % (Carius); this high figure agrees with the finding of a relatively large quantity of cystine in the molecule (see next paragraph). Taken in conjunction with the results shortly to be described for the ratio lecitho-vitellin : livetin in the yolk, and for the sulphur content of the former protein, it appears that about one-half of the total sulphur of the yolk is originally present as livetin.

2. *Amino-acids.* Using the method of Folin and Ciocalteu [1927] for tyrosine and tryptophan and that of Folin and Looney [1922] for cystine, the following values have been obtained for the percentage of these amino-acids in purified livetin (three specimens):

tyrosine 5.14, 5.20, 5.17; mean 5.17;

tryptophan 2.08, 2.34, 2.00; mean 2.14;

cystine 3.5, 4.3, 3.91; mean 3.9 (= 1.05 % cystine-sulphur).

According to analyses made at the same time, vitellin contains 5.0 % tyrosine, 1.6 % tryptophan and only 1.4 % cystine, with a total sulphur content of 0.6 %. Lecitho-vitellin contains only 0.5 % sulphur. Plimmer's suggestion of the possibility of a direct relationship between vitellin and livetin is not borne out by these results.

<sup>1</sup> Specimen of livetin from yolk of duck's egg.

*Physical constants.*

A preliminary investigation of some of the physical constants of the pure, dialysed protein has been made.

1. *The  $c_H$  for minimum solubility.* This was determined approximately by adding small portions of the protein solutions to acetate buffers, and then adding neutral alcohol till precipitation occurred. Using 2 cc. buffer, 0.3 cc. of 3.2 % livetin solution and 1 cc. alcohol, maximum precipitation occurred between  $p_H$  4.8 and 5.2. A second experiment with the same livetin solution gave optimal precipitation at  $p_H$  5.0. A different specimen of the protein (2.1 % solution) gave minimum solubility at  $p_H$  4.8<sup>1</sup>. It may therefore be concluded that the isoelectric point of livetin is in the neighbourhood of  $p_H$  4.8–5.0. The titration curve of the purified protein, kindly determined by Dr J. R. Marrack, indicates a broad isoelectric zone in this region.

2. *The refractive index* of the aqueous solution was determined using Pulfrich's dipping refractometer and a glass mercury-vapour lamp. At 20°, four alcohol-ether-extracted specimens gave 0.00184; 0.00192; 0.00189; 0.00196; mean 0.00190, as the refractive index for 1 % protein. Pure ovalbumin in the same apparatus gave a mean value for 1 % protein of 0.00192.

3. *The optical rotation* of several specimens of the protein, prepared by slightly different methods, and in solutions of different concentrations was kindly determined for us by Dr L. F. Hewitt. The specific rotation varied considerably with the purity of the material; for pure, alcohol-ether-extracted specimens the following results were obtained:

$[\alpha]_{5461}^{20}$  = (to nearest degree)  $-55^\circ$ ;  $-53^\circ$ ;  $-56^\circ$ ;  $-58^\circ$ ; mean  $-55.5^\circ$ .

The pure protein appears, therefore, to have a slightly higher specific rotation than ovalbumin, and a rather lower one than serum-globulin [cf. Hewitt, 1927, Table V].

## QUANTITY OF LIVETIN PRESENT IN THE YOLK.

A method for the estimation of the amount of livetin in a single yolk has been devised. The yolk is cleaned and dried as previously described, then transferred to a 50 cc. cylinder, the vitelline membrane being removed with forceps. (If absolute values are required, it is necessary to introduce obvious modifications here.) The volume is measured, an equal volume of 8 % NaCl is added, and the whole stirred vigorously till the mixture is homogeneous. 20 cc. of the diluted egg-yolk is transferred to a graduated, stoppered cylinder, and shaken with 40 cc. ether containing 2.5 % alcohol. The ether is removed after the layers have separated, and the extraction repeated thrice with 20 to 25 cc. of ether. The ether is removed as completely as possible and 4 % NaCl

<sup>1</sup> It is of interest to record that similar concentrations of pure ovalbumin, in these buffer solutions, required only one-fifth of this amount of alcohol to produce a turbidity; i.e. ovalbumin, though less readily precipitated by salt solutions, is much more sensitive to the presence of alcohol than is livetin.

solution added to make the volume up to 25 cc. 0.5 cc. is removed in duplicate for total N determination (micro-Kjeldahl) (*A*). 20 cc. are diluted to 200 cc. with distilled water. After standing overnight to allow the precipitated lecitho-vitellin to settle, the supernatant liquid is filtered. The protein in this filtrate is almost exclusively livetin. 20 cc. are taken in duplicate for total N determination (*B*). 80 cc. are treated with 20 cc. 25 % trichloroacetic acid, and filtered after standing for 15 minutes; total N is determined in duplicate on 40 cc. of the filtrate (*C*). The livetin in the original yolk may readily be calculated from the difference between *B* and *C*, whilst the difference between *A* and *B* gives the lecitho-vitellin content. (Livetin is assumed to contain 15 % and lecitho-vitellin 13 % N.)

By this method, the following values have been found:

Egg	Ratio lecitho-vitellin : livetin
Hen	3.83 : 1, 3.46 : 1, 3.89 : 1, 3.56 : 1, 3.62 : 1, 3.40 : 1; mean 3.63 : 1
Duck	3.96 : 1, 3.99 : 1, 3.35 : 1; mean 3.76 : 1

With the duck's egg the separation of the ether extract in the first stages of the determination is distinctly more difficult than with the hen's egg. A trace of capryl alcohol and the device of keeping the long extraction cylinder horizontal assist the separation.

It will be observed that, making allowance for certain almost inevitable experimental errors, the ratio lecitho-vitellin : livetin appears to be fairly constant from one egg to another, and possibly from one species to another. In the values given above for hens' eggs, the first four figures were from commercial "new-laid" eggs selected at random, the last two figures were from eggs 24 hours old, of pedigree Rhode Island Red parentage.

Between one-quarter and one-fifth of the protein of the yolk of the egg, therefore, is livetin, the absolute amount being, for an egg of average size, 0.6 to 0.9 g.

#### *Is livetin a single protein?*

Throughout this paper it has been tacitly assumed that the livetin of egg-yolk, prepared in the manner described, is a single individual. The method given above for determining the livetin in a single egg-yolk presupposes, in fact, that all the non-vitellin protein in egg-yolk is the same substance—livetin.

As against this assumption, it must be pointed out that (*a*) there is a small amount of protein precipitated by 25 % saturation with ammonium sulphate; (*b*) although there is only a trace of protein in the filtrate from 50 % ammonium sulphate precipitation, there appears to be no very sharp zone of precipitation between 25 and 50 % saturation in which the rest of the protein comes down quantitatively; (*c*) on long continued dialysis it appears possible to separate the livetin into two fractions, one more soluble in water than the other. So far the fractions have not been examined separately, but further work on this point is in hand.

In view of the work of Sørensen [1925] and of Linderström-Lang [1925], who have separated serum-euglobulin and pseudoglobulin and caseinogen into

fractions having different solubilities and phosphorus contents, the whole of our criteria as to the individuality of proteins may have to be revised. For the present it is justifiable to say that the preliminary examination of the non-vitellin protein of egg-yolk has revealed that by far the greater portion of it (90 % or more) behaves, by the usual criteria adopted up to now for protein characterisation, as a single individual.

#### SUMMARY.

1. It is confirmed that another protein beside vitellin is present in the yolk of the hen's or the duck's egg. This protein has been isolated in a fairly pure, undenatured form for the first time.

2. This substance, for which the name livetin suggested by Plimmer is retained, has, on the whole, the properties of a pseudoglobulin. It contains 5.2 % tyrosine, 2.1 % tryptophan, 3.9 % cystine, 0.05 % phosphorus, and 1.8 % sulphur. It has its isoelectric point in the neighbourhood of  $p_H$  4.8–5.0, refractive index in 1 % aqueous solution = 0.00190 at 20°,  $[\alpha]_{5461}^{20} = -55.5^\circ$ . It is probably unrelated to ovalbumin or vitellin. Its high content of the indispensable amino-acids cystine and tryptophan suggests that it may have a considerable biological value.

3. A method is described for estimating the amount of this protein in a single egg-yolk. In the fresh yolk, the ratio vitellin : livetin is fairly constant from one egg to another. Between one-quarter and one-fifth of the yolk-proteins of the hen's or duck's egg is livetin.

This work has been carried out with the aid of grants from the Research Fund of the London Hospital Medical College.

#### REFERENCES.

- Folin and Ciocalteu (1927). *J. Biol. Chem.* **73**, 627.  
Folin and Looney (1922). *J. Biol. Chem.* **51**, 527.  
Gross (1899). Dissertation, Strassburg.  
Hewitt (1927). *Biochem. J.* **21**, 216.  
Linderström-Lang (1925). *Compt. Rend. Trav. Lab. Carlsberg*, **16**, (i), 48.  
Plimmer (1908). *J. Chem. Soc.* **93**, 1500.  
Sørensen (1925). *J. Amer. Chem. Soc.* **47**, 457.

## CLVIII. CONSTITUTION OF HEXOSE-DIPHOSPHORIC ACID. PART II.

### THE DEPHOSPHORYLATED $\alpha$ - AND $\beta$ -METHYLHEXOSIDES<sup>1</sup>.

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*(Received September 7th, 1928.)*

THE researches of Harden and Young [1909, 1910] showed that the three monosaccharides glucose, mannose and fructose behave in the same way towards yeast juice in the presence of added phosphate. The identity of the hexosediphosphoric acid produced in each case was established by Young [1909], who showed that this ester on acid hydrolysis yielded free phosphoric acid and a laevo-rotatory solution from which fructose could be isolated. The rotation of this solution, however, was always lower than that of a fructose solution of equal reducing power, and this caused Young to avoid drawing definite conclusions as to the nature of the sugar in hexosediphosphoric acid. Later Neuberg and Kretschmer [1911] succeeded in isolating crystalline fructose from the products of hydrolysis and they concluded that this was the only sugar present and that the original acid was fructosediphosphoric acid.

At this time the reactive  $\gamma$ -sugars had not been described, for it was not until some years later that Fischer [1914] prepared  $\gamma$ -methylglucoside, the first representative of the structural isomerides containing an unstable oxide ring. The recognition that inulin and sucrose are derivatives of  $\gamma$ -fructose followed from the work of Haworth [1920] and Irvine and Steele [1920] and in 1922 Menzies [1922] isolated  $\gamma$ -methylfructoside from the products of esterification of ordinary fructose by Fischer's method.

In the light of this more recent knowledge of structural isomerism in the sugar group the further investigation of the constitution of hexosediphosphoric acid was undertaken in order to determine the type of oxide ring in the hexose molecule as well as the position of the phosphoric acid groups. Of these two groups the position of one followed with considerable certainty from the work of Young [1911] on the reaction of hexosediphosphoric acid with phenylhydrazine. Using cold reagents he obtained the phenylhydrazine salt of a

<sup>1</sup> The conclusions reached in this paper were communicated to the Biochemical Society on December 9th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, 46, 1183.

phenylhydrazone in which both phosphoric acid groups were still present. On heating, the osazone was formed, one phosphoric acid group however being simultaneously eliminated. From these facts Young concluded that if the parent hexose is fructose one phosphoric acid group is attached to the terminal carbon atom adjacent to the carbonyl group. No evidence was forthcoming as to the position of the second group.

The preparation of methylhexosidediphosphoric acid and the separation of the  $\alpha$ - and  $\beta$ -isomerides by crystallisation of their brucine salts has already been described [Morgan, 1927] and in the present paper experimental proof is given that the methylhexosidediphosphoric acids are derivatives of  $\gamma$ -fructose and the bearing of this conclusion on the constitution of the free phosphoric ester is discussed.

*The removal of the phosphoric acid groups from the  $\alpha$ - and  $\beta$ -methylhexosidediphosphoric acids by the bone phosphatase.*

By the action of the bone phosphatase it has been possible to eliminate the phosphoric acid groups from both the  $\alpha$ - and  $\beta$ -methylhexosidediphosphoric acids without removing the glucosidic methyl group, thus confirming and extending the preliminary experiment of Morgan [1927]. For most of the experiments here described we have used active preparations of the bone phosphatase purified by precipitation from the aqueous extract of bones of young rabbits by a mixture of ether and alcohol [Martland and Robison, 1928] and have in this way avoided contamination of the reaction products with alcohol-soluble material derived from the bones.

The barium salts of the methylhexosidediphosphoric acids were dissolved in water and treated with the bone phosphatase preparation. The flasks containing the mixture were immersed in a water-bath at  $38^{\circ}$  and the  $p_H$  of the solution during the hydrolysis was maintained at 8.6-8.8 by the addition of cold saturated baryta at short intervals. Under these conditions the inorganic phosphate liberated by the action of the enzyme was at once precipitated as barium phosphate. The progress of the hydrolysis was roughly indicated by the amount of baryta added but in practice this amount was usually considerably less than the equivalent of the inorganic phosphate set free, probably owing to the precipitation of a portion of the latter as  $BaHPO_4$  in place of  $Ba_3(PO_4)_2$ . For example, 0.6 g. bone enzyme acting on 4 g. barium methylhexosidediphosphate set free about 95 % of the phosphoric acid in 12 hours while the amount of baryta added corresponded with no more than 70 %. At the close of the hydrolysis the enzyme and any barium salts remaining in solution were precipitated by the addition of three volumes of alcohol and the alcoholic filtrate was evaporated to dryness *in vacuo*. The syrupy residue was dissolved in absolute alcohol and the filtered solution again evaporated to dryness. A blank experiment was in all cases carried out with the enzyme preparation alone but the amount of the alcohol-soluble matter so obtained did not exceed 2 % of the original weight of the enzyme pre-



paration. The small residues obtained in these blank experiments were optically inactive and possessed only very slight reducing properties either before or after acid hydrolysis.

The results of a number of experiments carried out on various specimens of  $\alpha$ - and  $\beta$ - and the mixed  $\alpha\beta$ -methylhexosidediphosphates are shown in Table I.

Table I.

Barium methylhexoside- diphosphate		Hydro- lysed by enzyme %	Dephosphorylated methylhexoside				[ $\alpha$ ] <sub>5461</sub> of hexoside	[ $\alpha$ ] <sub>5461</sub> of hexose formed by acid hydrolysis
			Reducing power mg. glucose	hydro- lysis mg. glucose	Equiv. weight of hexoside mg.	MeO in dried hexoside syrup %		
Isomer	[ $\alpha$ ] <sub>5461</sub>							
$\alpha$	+ 8.3°	—	4.0	83	89	—	+ 64°	- 103°
$\alpha$	+ 8.9°	98	0.0	233	251	—	+ 54°	—
$\alpha$	+ 8.3°	—	7.0	298	321	—	+ 48°	—
$\beta$	- 10.4°	—	0	280	302	14.7	- 61°	- 109°
$\beta$	- 8.3°	98	0	274	295	—	- 44°	—
$\beta$	- 9.7°	—	2.0	65	70	—	- 47°	- 101°
$\beta$	- 9.0°	—	1.2	85	92	—	- 44°	—
$\alpha\beta$	—	91	3.6	782	848	15.2	—	- 109°
$\alpha\beta$	—	96	8.4	846	910	15.6	—	—
Methyl fructoside requires			—	—	—	15.9	—	- 110.8° (fructose)

A small portion of the syrupy hydrolysis product was dried to constant weight *in vacuo* at 78° over phosphorus pentoxide and determinations of the methoxy-group were made on this material by the micro-Zeisel method. The specific rotations were determined on aqueous solutions of the partially dried syrups, the weight of hexoside being calculated from the reducing power (Hagedorn and Jensen) after acid hydrolysis. A small error is introduced by this method of calculation owing to the slight destruction of sugar which occurs during the hydrolysis in spite of the rapidity with which the latter takes place. This is shown by the following figures obtained for the reducing power after heating the  $\alpha$ - and  $\beta$ -methylhexosides with 0.1 *N* hydrochloric acid at 100° for varying periods.

*Reducing power as mg. glucose.*

	Time of heating			
	0 min.	3 min.	5 min.	10 min.
$\alpha$ -Methylhexoside	0	233	227	215
$\beta$ -Methylhexoside	0	274	271	264

The small reducing power shown by some of the syrups before acid hydrolysis and due in part to material derived from the enzyme preparation has also been neglected in these calculations. In view of these possible errors and of the variations in the specific rotations of the original barium methylhexosidediphosphates (see Table I) the values for the specific rotation of the methylhexosides show fair agreement.

The sensitiveness of the methylhexosides to acids is more clearly seen in Table II which shows the progress of hydrolysis of the  $\alpha$ - and  $\beta$ - and of the mixed  $\alpha\beta$ -isomers by 0.01 *N* acid at room temperature. The final values obtained for the specific rotation of the hexose thus formed are in satisfactory agreement with that of  $\alpha\beta$ -fructose (normal),  $[\alpha]_{5461} = -110.8^\circ$ . The extreme ease of hydrolysis and the specific rotation of the methylhexosides suggested, however, that the latter were not derivatives of the normal but of  $\gamma$ -fructose.

Table II. *Hydrolysis of methylhexosides with 0.01 N HCl.*

$\alpha$ -Methylhexoside vol. 10 cc., temp. 18°			$\beta$ -Methylhexoside vol. 10 cc., temp. 18°			Mixed $\alpha\beta$ -hexoside vol. 15 cc., temp. 22-23°		
Time (hours)	$\alpha$ observed ( $l = 1$ )	Reducing power as glucose mg.	$\alpha$ observed ( $l = 1$ )	Reducing power as glucose mg.	Time (hours)	$\alpha$ observed ( $l = 2$ )	Reducing power as glucose mg.	
0	+0.19°	1.2	-0.13°	0.17	0	+0.05	0	
4	+0.16°	—	—	—	4	+0.01	1.35	
20	+0.12°	5.4	-0.16°	2.5	23	-0.08	6.8	
27	+0.05°	6.3	-0.17°	3.1	49	-0.15	—	
44	+0.02°	9.3	-0.18°	4.8	72	-0.19	12.3	
92	-0.04°	14.8	-0.19°	8.5	96	-0.225	—	
116	-0.09°	—	-0.19°	—	120	-0.245	—	
140	-0.13°	—	-0.20°	—	168	-0.29	19.8	
208	-0.19°	—	-0.215°	—	192	-0.29	19.8	
280	-0.25°	26.4	-0.23°	17.5				
336	-0.29	29.2	-0.24°	22.0				
$[\alpha]_{5461}$ of hexose = -99°			$[\alpha]_{5461}$ of hexose = -109°			$[\alpha]_{5461}$ of hexose = -109°		

Irvine and Patterson [1922] and Menzies [1922] showed that  $\beta$ -methylfructoside when dissolved in 0.009 *N* hydrochloric acid at room temperature possessed a constant rotation for several days, while under parallel conditions the rotation of methyl- $\gamma$ -fructoside changed from dextro ( $[\alpha]_D + 22^\circ$ ) to laevo in 30 hours and reached the value  $[\alpha]_D - 64^\circ$  in 27 days. The specific rotations of the various methylfructosides recorded in the literature so far as we have been able to ascertain are given in Table III. The figures in brackets give the values of  $[\alpha]_{5461}$  calculated from those for  $[\alpha]_D$  by means of the factor 1.18.

Table III.

	$[\alpha]_D$	$[\alpha]_{5461}$	Reference
$\alpha$ -Methylfructoside (normal)	+45°	(+53°)	Schlubach and Schröter [1928]
$\beta$ -Methylfructoside (normal)	-172°	(-203°)	Hudson and Brauns [1916]
$\alpha$ -Methyl- $\gamma$ -fructoside	+65 to +70°*	—	Schlubach and Rauchsallies [1925]
$\beta$ -Methyl- $\gamma$ -fructoside	-17°*	—	Schlubach and Rauchsallies [1925]
$\alpha\beta$ -Methyl- $\gamma$ -fructoside	+26°	(+31°)	Menzies [1922]
$\alpha$ Methylhexoside from methylhexosidediphosphoric acid	—	+55° (mean)	Present communication
$\beta$ -Methylhexoside from methylhexosidediphosphoric acid	—	-47° (mean)	Present communication

\* These values were calculated from results of experiments on the hydrolysis of  $\alpha\beta$ -methyl- $\gamma$ -fructoside by invertase and are not claimed by the authors as more than approximations to the correct figures.

*Tetramethylmethylhexosides.*

Further confirmation that the  $\alpha$ - and  $\beta$ -methylhexosides possessed the  $\gamma$  or butylene oxide ring was obtained by converting them into the corresponding fully methylated tetramethyl- $\alpha$ - or - $\beta$ -methylhexoside by the action of silver oxide and methyl iodide [Purdie and Irvine, 1903]. These methylated derivatives were then converted into the tetramethylhexose by removal of the glucosidic methyl group by hydrolysis with dilute acid.

The very small quantities of the original methylhexosides, the poor yields obtained in the methylation and the unstable nature of the products added considerably to the difficulty of obtaining the tetramethylhexose in sufficiently pure condition for identification. Five successive methylations were carried out, in the first two of which a small quantity of methyl alcohol was added as an extraneous solvent. In the subsequent methylations the material was soluble in methyl iodide.

We did not succeed in raising the percentage of methoxyl in the crude methylated product above 50 % against a theoretical value of 62 % for tetramethylmethylhexoside. It was, however, found possible to purify these syrups by fractional distillation under pressures of about 1 mm., and although, when applied to such small quantities, this method could not be expected to yield perfectly pure products, a considerable improvement was effected, as can be seen from the percentage of  $\text{CH}_3\text{O}$  in the distillate (Table IV).

Table IV.

Hexoside methylated	$\text{CH}_3\text{O}$ before distillation %	$\text{CH}_3\text{O}$ after distillation %	Fraction collected	Weight of distillate mg.
$\alpha$	44.6	50.3	—	38
$\beta$	49.6	56.8	100-110° (1-1.5 mm.)	88
$\beta$	49.6	54.8	—	54
$\alpha\beta$	49.8	61.7	87-100° (0.5 mm )	180

*Hydrolysis of the tetramethylmethylhexosides.*

The fully methylated non-reducing hexosides were converted into the tetramethylhexose by hydrolysis with dilute mineral acid. The compounds were dissolved in water and concentrated hydrochloric acid was added to give a 0.1 *N* solution. The rotation became constant after heating for 3-5 minutes at 100°, showing that hydrolysis was complete. Table V gives the values of the specific rotations of the tetramethylmethylhexosides together

Table V.

Methylated product derived from	Before hydrolysis (tetramethylmethylhexoside)	After hydrolysis (tetramethylhexose)
	$[\alpha]_{\text{D}}^{25}$	$[\alpha]_{\text{D}}^{25}$
$\alpha$ -Hexoside	+ 15.5°	+ 25.6°
$\beta$ -Hexoside	- 6.8°	+ 31.9°
$\alpha\beta$ -Hexoside	- 1.3°	+ 40°

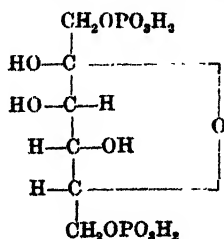
with those of the tetramethylhexose produced on hydrolysis. The three values for the tetramethylhexose should be identical since on removing the glucosidic methyl group the equilibrium mixture of  $\alpha$ - and  $\beta$ -forms would be obtained; but the purity of the products probably varied with the amount of the tetramethylmethylhexoside distilled, this amount being least in the case of the  $\alpha$ -compound and greatest in the case of the  $\alpha\beta$ -mixture. The tetramethylhexose obtained from the latter gave a value of 52.1 %  $\text{CH}_3\text{O}$  against 52.5 % calculated for tetramethylfructose. The specific rotation of this product ( $[\alpha]_{5461} + 40^\circ$ ) is in close agreement with that found by Irvine and Steele [1920] and Haworth [1920] for tetramethyl- $\gamma$ -fructose ( $[\alpha]_D + 31^\circ$ ) but widely different from the specific rotation of crystalline (normal) tetramethylfructose ( $[\alpha]_D - 121^\circ$ ; [Purdie and Paul, 1907]). From these results we therefore conclude that the isomeric methylhexosides prepared from hexosediphosphoric acid were derivatives of  $\gamma$ -fructose.

*Constitution of hexosediphosphoric acid.*

The formation of methyl- $\gamma$ -fructoside from hexosediphosphoric acid does not at once prove that the latter compound is itself a derivative of  $\gamma$ -fructose, since it has been shown by Purdie and Paul [1907] and Menzies [1922] that ordinary fructose, esterified by Fischer's method, yields a mixture of normal and  $\gamma$ -methyl derivatives. However, the  $\alpha\beta$ -methylhexoside which yielded the purest tetramethyl- $\gamma$ -fructose ( $[\alpha]_{5461} + 40^\circ$ ) represented about 80 % of the theoretical yield from the original hexosediphosphoric acid and neither in this nor in other preparations was evidence obtained of other isomers of different properties which might correspond with the more stable normal derivatives.

This would seem to indicate that the free acid also possesses the  $\gamma$ -fructose structure but it is hoped that further work now in progress will yield more conclusive evidence on this point.

The presence of the butylene oxide ring in the methylhexosidediphosphates implies that neither of the phosphoric acid groups can be in position 5; while, if the presence of a similar oxide ring in the free hexosephosphoric acid is accepted, we may infer that one phosphoric acid group is in position 6 since if this terminal  $\text{CH}_2\text{OH}$  group were unsubstituted we should expect the stable amlene oxide ring to be formed. Accepting Young's conclusion as to the position of the other phosphoric acid group we suggest therefore that hexosediphosphoric acid is  $\gamma$ -fructose-1 : 6-diphosphoric acid.



If this structure proves to be correct hexosediphosphoric acid will fall into line with other naturally occurring fructose derivatives, sucrose, raffinose, inulin, in which the fructose has been shown to possess the unstable butylene oxide ring.

#### SUMMARY.

1. By the action of the bone phosphatase on the  $\alpha$ - and  $\beta$ -methylhexoside-diphosphoric acids the corresponding  $\alpha$ - and  $\beta$ -methylhexosides have been obtained as non-reducing syrups.

2. The properties of these compounds agree with those expected for  $\alpha$ - and  $\beta$ -methyl- $\gamma$ -fructosides.

3. These methylhexosides have been further converted into fully methylated derivatives from which tetramethyl- $\gamma$ -fructose has been obtained by acid hydrolysis.

4. The bearing of these facts on the constitution of hexosediphosphoric acid is discussed and it is suggested that this compound is  $\gamma$ -fructose-1 : 6-diphosphoric acid.

#### REFERENCES.

- Fischer (1914). *Ber. deutsch. chem. Ges.* **47**, 1980.  
Harden and Young (1909). *Proc. Roy. Soc. Lond.* B, **81**, 336.  
—— (1910). *Proc. Roy. Soc. Lond.* B, **82**, 321.  
Haworth (1920). *J. Chem. Soc.* **117**, 207.  
Hudson and Brauns (1916). *J. Amer. Chem. Soc.* **38**, 1216.  
Irvine and Patterson (1922). *J. Chem. Soc.* **121**, 2703.  
Irvine and Steele (1920). *J. Chem. Soc.* **117**, 1487.  
Martland and Robison (1928). (In the Press.)  
Menzies (1922). *J. Chem. Soc.* **121**, 2245.  
Morgan (1927). *Biochem. J.* **21**, 675.  
Neuberg and Kretschmer (1911). *Biochem. Z.* **36**, 5.  
Purdie and Irvine (1903). *J. Chem. Soc.* **83**, 1021.  
Purdie and Paul (1907). *J. Chem. Soc.* **91**, 289.  
Schlubach and Rauchalles (1925). *Ber. deutsch. chem. Ges.* **58**, 1842.  
—— and Schröter (1928). *Ber. deutsch. chem. Ges.* **61**, 1216.  
Young (1909). *Proc. Roy. Soc. Lond.* B, **81**, 528.  
—— (1911). *Biochem. Z.* **32**, 178.

# CLIX. TREHALOSEMONOPHOSPHORIC ESTER ISOLATED FROM THE PRODUCTS OF FERMENTATION OF SUGARS WITH DRIED YEAST.

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*(Received September 12th, 1928.)*

SINCE the original work of Harden and Young on the function of phosphates in alcoholic fermentation, the idea that the formation of phosphoric esters may be an essential stage not only in the biochemical degradation of hexoses but also in the condensation of these sugars to the polysaccharides has taken firm hold of biochemical imagination. The experimental evidence, which consists in (a) the stimulating effect of phosphates on certain processes involving carbohydrates in living tissues or tissue extracts, (b) the occurrence of phosphoric esters among the reaction products, is admittedly insufficient to prove such a general conception, but this remains a stimulating basis for research. As to the manner in which the introduction of the phosphoric acid group furthers such reactions we have, as yet, very little knowledge.

Of the two phosphoric esters hitherto isolated from the products of alcoholic fermentation, hexosediphosphoric acid is probably the 1:6-diphosphoryl derivative of  $\gamma$ -fructose [Young, 1909, 1911; Morgan, 1927; Morgan and Robison, 1928], although the proof of this structure is not yet complete. The constitution of the hexosemonophosphoric acid is still unknown but certain facts set out in the account of its isolation and investigation [Robison, 1922] point to it being a mixture of monophosphoric esters of glucose and fructose. This view is supported by the evidence [Meyerhof and Lohmann, 1927] of its reducing power towards sodium hypoiodite, a reagent which distinguishes between aldoses and ketoses. It is possible that one of the components may prove to be identical with the monophosphoric ester obtained by Neuberg [1918] by the partial hydrolysis of hexosediphosphoric ester by acids and by the enzymes of *Aspergillus oryzae* and of yeast [Neuberg and Leibowitz, 1927, 1, 2].

During the isolation of hexosemonophosphoric acid from the products of the action of yeast juice on hexoses indications were obtained of the presence of other esters, in particular of a compound with properties intermediate between those of the mono- and di-phosphates, but the results of experiments

carried out at that time were not sufficiently conclusive to warrant publication. During the past two years this and other points of interest arising out of the early work have been reinvestigated, employing micro-methods of analysis which have made it possible to follow the progress of the fractionations at every stage. The full account of these experiments will be given in a later communication. The present paper is confined to the description of a new ester, entirely distinct from the compound referred to above, which has been isolated from the products of the fermentation of hexoses by dried yeast in presence of phosphates. This ester has proved to be a monophosphoric ester of the non-reducing disaccharide trehalose, a glucose glucoside found in trehala manna (excreted by the insect *Larinus maculatus*), in certain fungi, seaweeds and yeasts.

A series of fermentation experiments was carried out with various samples of yeast juice, dried yeast and zymin, using both fructose and glucose but otherwise keeping the conditions as similar as possible. The products were worked up according to a definite scheme, somewhat modified from the original method [Robison, 1922] as further experience had suggested. The separate experiments were carried out using 50 g. of dried yeast or zymin, or 300 cc. of yeast juice, to which were added 100 g. glucose or fructose, and sodium phosphate equivalent to about 7 g. P. The phosphate solution was added in six equal portions over a period of 5 to 6 hours, as indicated by the rate of evolution of carbon dioxide. When, after the last addition, the rate had again fallen, trichloroacetic acid was added to the fermentation mixture in amount equal to 4 % of the total volume. The precipitated proteins were filtered off and the filtrate treated with barium acetate equal in weight to the crystalline sodium phosphate originally added, followed by baryta in hot aqueous solution until pink with phenolphthalein. The precipitate consisting of crude barium hexosediphosphate was removed and a further precipitate, obtained by adding one-tenth volume of alcohol to the filtrate, was likewise filtered off. The solution containing the soluble barium salts was treated with basic lead acetate, and the precipitated lead salts decomposed with hydrogen sulphide. After removal of the excess of hydrogen sulphide by aeration, the acid solution was neutralised with baryta and poured into 3 volumes of alcohol. By extracting the fraction with 10 parts of 10 % aqueous alcohol the readily soluble barium salts were freed from a further quantity of sparingly soluble compounds.

All fractions obtained at each step in the separation were quantitatively examined, the total and inorganic phosphorus, the reducing power by the Hagedorn and Jensen and by the sodium hypiodite methods, and the specific rotation being measured.

On comparison of the results obtained for the whole series of experiments a very striking difference was seen between the products of fermentation with yeast juice on the one hand and with dried yeast and zymin on the other. In the experiments with yeast juice the sparingly soluble barium hexosediphosphate represented less than half the total esterified phosphorus, while

the properties of the large, readily soluble fraction were similar to those of the barium hexosemonophosphate previously described. As in the earlier experiments fractions of intermediate solubility were also obtained and these are being investigated. In the experiments with dried yeast and zymin a much higher proportion of diphosphate was obtained, this fraction representing between 80 and 90 % of the total esterified phosphorus. Further, the small fraction of readily soluble salt differed markedly in its properties from barium hexosemonophosphate, as is shown in Table I, in which the results of a number of experiments with dried yeast and zymin are summarised. One yeast juice experiment is included for purposes of comparison.

Table I. *Readily soluble fraction of barium salts.*

Exp.	Type of yeast preparation*	Sugar	Total P in fraction as percentage of total esterified P†	P %	Reducing power as glucose		Reduction $[\alpha]_{540.1}$	P	$[\alpha]_{540.1}$ P
					H. & J %	Iodine %			
6	Dried yeast	Fructose	5.9	5.5	8.3	12.5	+56.7°	1.5	10.4
7	"	"	11.7	6.3	13.6	15.6	+41.4°	2.2	6.6
8	"	Glucose	10.7	5.9	11.0	14.1	+56.4°	1.9	9.6
9	"	Fructose	6.4	5.3	5.5	10.8	+63.8°	1.0	12.0
15	Zymin	"	7.5	6.7	18.1	20.0	+28.0°	2.7	4.2
16	"	Glucose	4.3	6.2	16.2	19.0	+30.0°	2.6	4.8
13	Yeast juice	Fructose	47.3	7.4	27.4	23.4	+11.4°	3.7	1.6

\* Exps. 6, 7 and 8 were carried out with English brewery top yeast. In Exp. 9 a bottom yeast obtained from the Standard Yeast Co. was used.

† A small quantity of organic phosphorus remained in the final filtrates from the basic lead and barium salts. Some of this could be recovered in a very impure condition by treatment of these filtrates with basic lead acetate and ammonia. These amounts, if credited to the most soluble fraction, would increase the figures shown in the above table by several per cent.

#### *Isolation of the new phosphoric ester.*

The values shown in Table I indicated that the soluble fractions from the dried yeast and zymin fermentations contained a compound possessing a much higher dextro-rotation and lower reducing power than those of barium hexosemonophosphate. It was found that on fractional precipitation of the aqueous solution by alcohol the specific rotation of the more soluble portion was still further raised and the reducing power lowered. Accordingly a quantity of the crude soluble barium salts (about 50 g.) obtained from 5 experiments (Nos. 1-5), in all of which dried English top yeast and fructose had been used, was dissolved in 10 parts of water and treated with alcohol, raising the concentration of the latter successively to 10, 17, 23, 30 and 70 %. The precipitates obtained between 10 and 30 % alcohol were oily in nature and as the solubility of these salts increased with the temperature it was found convenient to heat the solution to about 50° before adding the alcohol, whereby the separation of an oily phase took place slowly during cooling to room temperature. After standing for some hours the clear supernatant solution could be readily poured off, again warmed and treated with a further quantity of alcohol. No advantage was gained by cooling the solution to 0° or by separating the fraction at 37°.



The small flocculent precipitate obtained on raising the concentration of alcohol from 30 to 70 % possessed a high dextro-rotation and very low reducing power. After many repetitions of this process a quantity of barium salt was obtained which possessed a reducing power (H. and J.) of only 1 % in terms of glucose whilst the specific rotation had risen to over + 100°. Since continued fractionation by alcohol did not further lower the reducing power the barium salt was converted into the brucine salt which unexpectedly proved to be very sparingly soluble in cold water and readily crystallisable from hot aqueous solution. After several recrystallisations it was reconverted into the barium salt which now showed no reducing power either by the Hagedorn and Jensen or the sodium hypoiodite method. The analytical figures for this salt agreed with those required for a disaccharide monophosphate, while the properties of the ester, its resistance to acid hydrolysis, negative Selivanov's reaction and the very high specific rotation ( $[\alpha]_{5461} + 132^\circ$ ) suggested that it was a derivative of trehalose. This supposition was confirmed by investigation of the products of hydrolysis of the ester by acids and by the bone phosphatase, the former yielding glucose and glucosemonophosphoric ester, while the latter yielded trehalose itself which was isolated in pure crystalline form.

*Brucine salt of trehalosemonophosphoric ester.*

The barium salt obtained from the crude soluble fraction by repeated precipitation with alcohol (30 to 70 % fractions) was dissolved in five parts of water and treated with the theoretical quantity of sulphuric acid. After removal of the barium sulphate by centrifuging, brucine (2 mols.) was added and the solution treated with an equal volume of acetone. The brucine salt crystallised out in the course of a few hours. It was recrystallised several times from hot water, from which it separated in large clusters of needles. It is very slightly soluble in hot ethyl alcohol, somewhat more soluble in methyl alcohol, insoluble in chloroform and acetone.

*Analysis.* For analysis the salt was dried over sulphuric acid at room temperature. The water of crystallisation was estimated by heating over  $P_2O_5$  at 100° and 15 mm. until the weight was constant (2 hours). P was estimated by the Briggs method; C and H by Pregl's micro-method.

	H <sub>2</sub> O	C	H	P
Found	11.8 11.7	50.9	6.8	2.31
Calculated for $C_{12}H_{21}O_{10}PO_4H_2(C_{22}H_{26}O_4N_2)_2 \cdot 9H_2O$	11.66	50.73	6.82	2.26
$[\alpha]_{5461}^{20^\circ}$ in water ( $c=0.77$ %) + 31.3°				
$[\alpha]_{5461}^{20^\circ}$ for the anhydrous salt + 35.4°				

*Barium trehalosemonophosphate.*

The pure brucine salt was dissolved in warm water and treated with a cold aqueous solution of baryta until pink with phenolphthalein. The barium salt was precipitated by the addition of 3 volumes of alcohol, filtered, washed with absolute alcohol and dried quickly in a vacuum desiccator. To remove traces

of brucine the salt was dissolved in 5 parts of water, reprecipitated with alcohol, filtered and dried as before, and the process repeated. The salt so prepared is a white amorphous powder, stable in the air, but like hexosemonophosphate turning syrupy with small quantities of water, in which it is very soluble. It is insoluble in absolute alcohol but in aqueous alcohol it is decidedly more soluble than barium hexosemonophosphate, the solubility increasing with the temperature. At 22°, 100 cc. of 30 % (by vol.) alcohol dissolves about 1 g. while the 75 % alcohol filtrates still contain appreciable traces of the salt. Unlike the hexosephosphates it can be heated to above 100° in the air or *in vacuo* without becoming discoloured, this stability being probably connected with absence of a reducing group. At 130° it slowly turns yellow. It does not reduce Hagedorn and Jensen's solution and gives no coloration with Selivanov's reagent for fructose.

*Analysis.* For analysis the salt was dried at 100° and 15 mm. over  $P_2O_5$ , or in Pregl's micro-desiccator at 120°, until the weight remained constant. *a*, *b* and *c* were different specimens, but were all derived from the same 50 g. of crude soluble salts. The results do not at present justify a definite conclusion as to the presence of a molecule of water in the salt.

	C	H	P	Ba
Found ( <i>a</i> )	25.5	4.0	5.43	23.6
	25.5	4.0	—	—
( <i>b</i> )	25.9	4.3	5.35	24.4
( <i>c</i> )	25.7	4.2	5.51	23.4
	25.6	4.1	5.49	23.4
	25.5	4.1	—	23.5
	25.6	4.1	—	—
Calculated for $C_{12}H_{21}O_{14}P\text{Ba}$	25.82	3.80	5.37	24.63
Calculated for $C_{12}H_{21}O_{14}P\text{Ba} + H_2O$	25.01	4.03	5.39	23.84
$[\alpha]_{5461}^{20^\circ}$ of the salt in water ( $c = 3.2\%$ )	+ 132°			
$[\alpha]_{5461}^{20^\circ}$ of the free acid in water ( $c = 2.4\%$ )	+ 185°			
$[M]_{5461}^{20^\circ}$ of the free acid ( $M = 422$ )	+ 781°			

*Hydrolysis of trehalosemonophosphoric ester by bone phosphatase.*

A preliminary experiment showed that bone phosphatase removed the phosphoric acid group of the new ester but was without action on the liberated disaccharide. Accordingly 380 mg. of the dry barium salt dissolved in 10 cc. water was treated with purified bone phosphatase (to be described shortly in a paper by Martland and Robison) and kept at 40°, the  $p_H$  being maintained at 8.6–8.8 by the addition of baryta at frequent intervals. Hydrolysis was complete in about 10 hours and alcohol (3 vols.) was then added to precipitate the enzyme and any barium salts remaining in solution. The alcoholic filtrate was evaporated in a vacuum desiccator and the syrupy residue taken up in methyl alcohol. The solution was filtered and evaporated to dryness, the residue dissolved in a few cc. of water and the solution again filtered and evaporated.

Weight of residue, 169 mg. representing 73 % of the theoretical yield of trehalose. It contained no phosphorus, was non-reducing, and had  $[\alpha]_{5461}^{20^\circ} + 196^\circ$ .

The product was further purified by recrystallisation from hot alcohol from which it separated in lustrous rhombic prisms, m.p.  $96^\circ$ – $98^\circ$  (uncorr.). This figure is in agreement with the m.p. of hydrated trehalose given by Schukoff [1900]. Koch and Koch [1925] give a somewhat higher m.p.,  $102.5^\circ$ . The solubilities of the crystals in water and alcohol also agreed with those of trehalose.

*Analysis.* For analysis the air-dried crystalline substance was used.

	C	H
Found	38.1	6.8
	38.0	6.8
Calculated for $C_{12}H_{22}O_{11}$ , $2H_2O$	38.1	6.9
$[\alpha]_{5461}^{19^\circ}$ in water ( $c=0.2$ %) + $222^\circ$		

The values of  $[\alpha]_D$  of trehalose recorded in the literature vary between  $+173^\circ$  (for the hydrated sugar) [Mitscherlich, 1858] and  $+184^\circ$  [Koch and Koch, 1925]. No figures for  $[\alpha]_{5461}$  have been found, but Koch's figure for  $[\alpha]_D$  multiplied by the factor 1.18 gives  $[\alpha]_{5461} + 217^\circ$ . Calculated from this figure the molecular rotation of trehalose is  $[M]_{5461} + 820^\circ$ , while that found for the free trehalose-monophosphoric ester was  $+781^\circ$ . The effect of the introduction of the phosphoric acid group on the rotatory power of the molecule is thus relatively small.

*Hydrolysis by acids.* The disaccharide was very resistant to acid hydrolysis. 16 mg. of the crystalline sugar were dissolved in 10 cc. 2 N  $H_2SO_4$  and heated at  $100^\circ$  until the rotation became approximately constant (5 hours). The initial and final values observed for rotation and reducing power are shown below:

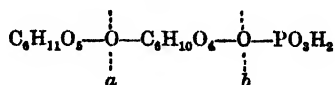
Time (hours)	$\alpha_{5461}$ ( $l=1$ )	Reducing power (mg. glucose in 10 cc.)	
		H. and J.	Iodine
0		0	0
5	$+0.35^\circ$ $+0.13^\circ$	13.6	13.2
Percentage of total change calculated for conversion to two molecules of glucose	86	88	86

An osazonewas prepared from the hydrolysis product, which, after recrystallisation from dilute pyridine, melted at  $202^\circ$ – $204^\circ$  and did not lower the melting-point when mixed with a specimen of glucosazone. The properties of the disaccharide obtained from the ester by enzymic hydrolysis thus agreed in all respects with those of trehalose.

#### *Hydrolysis of trehalosemonophosphoric ester by acid.*

Trehalosemonophosphoric ester is slowly hydrolysed by dilute mineral acids at  $100^\circ$ , the ultimate products being glucose and phosphoric acid. Since

the rate of hydrolysis of the phosphoryl linkage "b" is very much slower than that of the glucosidic linkage "a"



glucose and glucosemonophosphoric ester at first accumulate and the latter can be isolated by stopping the hydrolysis at a suitable stage. Owing to the small quantity of material available, the glucosemonophosphate has not yet been obtained in absolutely pure condition but it will be interesting to discover whether, as appears likely, it is identical with one constituent of hexosemonophosphate (Robison).

Table II.

Hydrolysis of trehalosemonophosphoric ester (*M*/20 sol.) by 0.2 *N* H<sub>2</sub>SO<sub>4</sub> at 100°. Degree of hydrolysis as measured by

Time (hours)	Inorganic P set free %	Reducing power		Fall in rotation %
		H. and J. %	Iodine %	
5	3	18	22	19
10	9	34	40	39
21	18	64	71	73
40	36	82	93	93
58	51	86	97	100
67	53	84	100	101

Table II shows the progress of one hydrolysis experiment with 0.2 *N* acid. Even after 67 hours only 53 % of the phosphoric acid had been set free. The reducing power towards an alkaline solution of iodine is a measure of the free aldose group and the figures in this column show that hydrolysis of the glucosidic linkage was complete. The lower values obtained by the Hagedorn and Jensen method are due to the presence of glucosemonophosphate which has only two-thirds the reducing power of the equivalent weight of glucose. The fall in rotation, also calculated as a percentage of the total change for complete hydrolysis to glucose, is seen to follow very closely the figures for the reducing power towards iodine, showing that the molecular rotation of the glucosemonophosphoric ester is very similar to that of glucose.

The results of another acid hydrolysis with *N* H<sub>2</sub>SO<sub>4</sub> are shown in Fig. 1, the parallelism between the increase in reducing power towards iodine (curve *C*) and fall in rotation (curve *D*) being again very marked. The divergence of the two curves towards the end of the experiment is due to the destruction of a little glucose by the acid which increases the apparent hydrolysis as measured by the fall in rotation. This also explains the fall in the reducing power by the Hagedorn and Jensen method. In this experiment the hydrolysis was stopped after 21 hours and the acid solution neutralised with baryta. After removal of the barium sulphate the solution was concentrated and poured into twice its volume of alcohol to precipitate the barium glucosemonophosphate. The

alcoholic filtrate on evaporation yielded a reducing sugar having all the properties of glucose.

Weight of product	...	...	...	...	...	...	0.524 g.
Reducing power as glucose (Hagedorn and Jensen)	...	...	...	...	...	...	0.393 g.
Reducing power as glucose (Iodine)	...	...	...	...	...	...	0.384 g.
[ $\alpha$ ] <sub>5461</sub> calculated on the glucose content as estimated by Hagedorn and Jensen's method							
For glucose [ $\alpha$ ] <sub>5461</sub>	...	...	...	...	...	...	+ 61°
Osazone: M.P. 204°, not lowered by admixture with pure glucosazone.							+ 62°

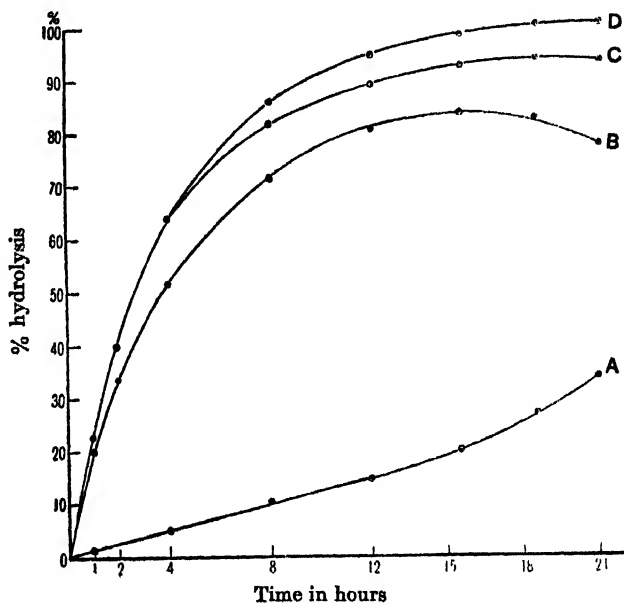


Fig. 1. Hydrolysis of trehalosemonophosphoric ester by  $N H_2SO_4$  at 100°.

Curve A. Inorganic P liberated.

Curve B. Reducing power by Hagedorn and Jensen method.

Curve C. Reducing power by the sodium hypoiodite method.

Curve D. Change in rotation.

The points on the curves represent in each case percentages of the total values calculated for complete hydrolysis of the ester to glucose (2 mols.) and phosphoric acid.

The alcohol precipitates obtained from this and other hydrolysis experiments were separately purified by solution in 10 % alcohol, and precipitation by addition of twice the volume of alcohol. Even after several repetitions of this process the salt still contained a small proportion of sparingly soluble substance. It was difficult to account for the persistence of this impurity which may have been due to oxidation or other change of the monophosphate. The figures for this compound must therefore be given subject to future correction.

*Barium glucosemonophosphate.*

Preparation	Total P %	Reducing power as glucose		[ $\alpha$ ] <sub>540</sub>
		H. and J. %	Iodine %	
<i>a</i>	7.63	27.9	35.6	+20°
<i>b</i>	7.40	32.0	42.0	+23.5°
<i>c</i>	7.33	31.0	42.4	+21°
Calculated for barium glucosemonophosphate	7.85	(132)	45.5	—

*Fermentation of trehalosemonophosphate.*

Experiments were made to determine whether trehalosemonophosphoric ester is fermented by yeast juice, dried yeast and zymin. A solution of the sodium salt (sol. *a*) was used, of such strength that 1 cc. was equivalent to 30 mg. glucose, and control tests were also made to determine (*b*) the rate of fermentation of sucrose (sol. *b*, 1 cc. = 40 mg. sucrose), and (*c*) the autofermentation rate of the yeast preparation.

1 cc. sol. *a* should give about 8 cc. carbon dioxide on complete fermentation. 1 cc. sol. *b* should give about 11.2 cc. carbon dioxide on complete fermentation.

The results show that the ester was readily fermented by dried yeast, the rate of decomposition being even more rapid than in the case of sucrose. It was also fermented, although more slowly, by zymin and by yeast juice.

- Exp. 1.* (*a*) 0.2 g. dried yeast + 1 cc. water + 1 cc. sol. *a* + 1 drop toluene.  
 (*b*) 0.2 g. dried yeast + 1 cc. water + 1 cc. sol. *b* + 1 drop toluene.  
 (*c*) 0.2 g. dried yeast + 2 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved		
	( <i>a</i> )	( <i>b</i> )	( <i>c</i> )
0	0.0	0.0	0.0
$\frac{1}{2}$	1.70	0.71	0.30
1	3.45	1.70	0.71
$1\frac{1}{2}$	4.40	2.30	0.80
3	5.69	3.59	0.90
5	5.91	5.35	0.95
22	6.47	6.66	0.95
Total corrected for autofermentation ( <i>c</i> )	5.52	5.71	—

- Exp. 2.* (*a*) 0.2 g. zymin + 1 cc. water + 1 cc. sol. *a* + 1 drop toluene.  
 (*b*) 0.2 g. zymin + 1 cc. water + 1 cc. sol. *b* + 1 drop toluene.  
 (*c*) 0.2 g. zymin + 2 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved		
	( <i>a</i> )	( <i>b</i> )	( <i>c</i> )
0	0.0	0.0	0.0
1	3.75	4.36	3.15
$2\frac{1}{2}$	6.90	11.36	6.30
18	11.97	17.86	7.42
Total corrected for autofermentation ( <i>c</i> )	4.55	10.44	—

Exp. 3. ( $a_1$ ) and ( $a_2$ ) 1 cc. yeast juice + 1 cc. sol. a + 1 drop toluene.  
 (c) 1 cc. yeast juice + 1 cc. water + 1 drop toluene.

Time (hours)	cc. carbon dioxide evolved		
	( $a_1$ )	( $a_2$ )	(c)
0	0.0	0.0	0.0
$\frac{1}{2}$	0.63	0.68	0.37
1	1.37	1.39	0.98
$1\frac{1}{2}$	2.07	2.17	1.50
15	6.65	7.01	2.37
Total corrected for autofermentation (c)	4.28	4.64	—

*Examination of dried yeast for trehalose and trehalosemonophosphate.*

Trehalose has been isolated from dried yeast by Koch and Koch [1925]. These workers obtained the sugar by extracting 40 lb. of air-dried yeast with 90–95 % alcohol but give no information as to the yield. From the data of our experiments it may be reckoned that the amount of trehalosemonophosphoric ester present in the fermentation products from 50 g. dried yeast, corresponded with at least 100–300 mg. P, i.e. about 1–3 g. of trehalose. It was not impossible that such amounts of the ester were originally present in the yeast or were produced during the fermentation by simple esterification of preformed trehalose. Experiments were carried out to test these possibilities.

(a) *Presence of trehalose in dried yeast.* 10 g. dried yeast was extracted for 8 hours with boiling ether to remove fat. It was then extracted with three successive quantities of boiling 96 % alcohol for 24 hours in all. The residues obtained on evaporating the three alcoholic extracts weighed 445 mg., 59 mg. and 38 mg., making a total of 542 mg. The rotation and reducing power of this residue before and after acid hydrolysis (2 N HCl, 100°, 6 hours) are shown below:

542 mg. in 30 cc. 2 N HCl	Before hydrolysis	After hydrolysis
$\alpha_{5461}^D (l = 1)$	– 0.02°	– 0.04°
Total reducing power as glucose	18 mg.	37 mg.

Even assuming that the increase in reducing power on hydrolysis was entirely due to trehalose, the amount of this sugar present in the yeast could not be more than about 0.2 %, equivalent to 100 mg. trehalose in 50 g. dried yeast. Other samples of dried yeast used in the fermentation experiments gave similar results.

(b) *Presence of trehalosemonophosphoric ester in dried yeast.* 50 g. of the dried yeast used in the fermentation experiments Nos. 1–5 were extracted with 250 cc. of 4 % trichloroacetic acid for 3 hours at room temperature. The extract, which contained 198 mg. inorganic P and 552 mg. organic P, was filtered and neutralised with baryta. The precipitated barium salts contained the whole of the inorganic P and over 92 % of the organic P, a large proportion of the latter being present as nucleic acid. Some hexosediphosphate was also present. The readily soluble fraction, in which barium trehalosemonophosphate would be found, contained only 15 mg. phosphorus and gave a strongly

laevorotatory solution. Two similar extractions of other dried yeast preparations gave very similar figures showing that trehalosemonophosphoric ester, if present in the original dried yeast, can only occur in very small amounts.

#### DISCUSSION.

From the experimental results given above it must be concluded that the trehalosemonophosphoric ester isolated from the products of the fermentation of fructose by dried yeast was not originally present in the yeast, nor could it have been derived to any great extent from preformed trehalose. The occurrence of a trehalase in various types of yeast has been observed [Fischer, 1895; Kalanthar, 1898; Bau, 1899; Lindner, 1911] and by the synthetic action of this enzyme, trehalose could be formed from glucose derived from the yeast glycogen. It is unlikely, however, that the dried yeast contained sufficient glycogen to account for more than a small fraction of the trehalosephosphoric ester, nor is this necessary since there is sufficient other evidence of the formation of glucose derivatives during the fermentation of fructose.

Trehalosemonophosphoric ester has not so far been found in the products of fermentation of yeast juice. It appears to be formed equally in the fermentation of fructose or glucose by dried yeast and also by zymin. (The investigation of the zymin products is not yet complete.) Using the same dried yeast preparation but varying the conditions of fermentation we have been able to modify the amount and nature of the soluble fraction of barium salts and are continuing the investigation on these lines.

It would be premature to venture an opinion as to whether the formation of this disaccharide phosphoric ester has any great significance in the carbohydrate metabolism of the yeast, but it is not impossible that the introduction of the phosphoric acid group may facilitate the building up of the complex reserve carbohydrates from the simple sugars.

Neuberg and Leibowitz [1928] have recently reported the preparation of another disaccharide monophosphoric ester by the action of *B. delbrücki* on hexosediphosphate. This ester, which was obtained in a yield of 30 %, possessed reducing properties and  $[\alpha]_D + 38^\circ$  for the barium salt. It is therefore not identical with the ester described in this paper. A synthetic trehalosemonophosphate has been prepared by Helferich, Löwa, Nippe and Riedel [1923] by the action of phosphoryl chloride on trehalose in pyridine solution. No properties are given other than the specific rotation of the acid barium salt,  $[\alpha]_D + 135.5^\circ$ , and it is therefore impossible to decide whether their compound was identical with the ester which has now been isolated from the products of fermentation.



## SUMMARY.

1. A monophosphoric ester of the disaccharide trehalose has been isolated from the products of fermentation of fructose by dried yeast, and has been purified by means of its barium salt, which is very soluble in water, and its crystalline brucine salt.

2. The ester is non-reducing and is strongly dextrorotatory:  $[\alpha]_{5461}$  of the free acid + 185°, of the barium salt + 132°, and of the brucine salt + 31°.

3. By the action of the bone phosphatase on the ester pure crystalline trehalose has been obtained.

4. Hydrolysis with boiling mineral acid slowly converted the ester into glucose and glucosemonophosphoric ester, the latter being still more slowly hydrolysed to glucose and phosphoric acid. Barium glucosemonophosphate was isolated but in a slightly impure condition.

5. Trehalosemonophosphoric ester is readily fermented by dried yeast and more slowly by yeast juice and zymmin.

6. Examination of the dried yeast used for the fermentation showed that neither trehalosemonophosphoric ester nor free trehalose was present in quantities sufficient to account for the amount of the ester found in the fermentation products.

7. No evidence has been obtained of the presence of this ester in the products of fermentation by yeast juice.

## REFERENCES.

- Bau (1899). *Woch. Brau.* **16**, 305.  
Fischer (1895). *Ber. deutsch. chem. Ges.* **28**, 1429.  
Helferich, Löwa, Nippe and Riedel (1923). *Z. physiol. Chem.* **128**, 141.  
Kalanthar (1898). *Z. physiol. Chem.* **26**, 88.  
Koch and Koch (1925). *Science*, **61**, 570.  
Lindner (1911). *Woch. Brau.* **26**, 612.  
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.  
Mitscherlich (1858). *J. prakt. Chem.* **73**, 70.  
Morgan (1927). *Biochem. J.* **21**, 675.  
Morgan and Robison (1928). *Biochem. J.* **22**, 1270.  
Neuberg (1918). *Biochem. Z.* **88**, 432.  
Neuberg and Leibowitz (1927, 1). *Biochem. Z.* **187**, 481.  
— (1927, 2). *Biochem. Z.* **191**, 460.  
— (1928). *Biochem. Z.* **193**, 237.  
Robison (1922). *Biochem. J.* **16**, 809.  
Schukoff (1900). *Z. Ver. deut. Zuckerind.* 818.  
Young (1909). *Proc. Roy. Soc. Lond. B*, **81**, 528.  
— (1911). *Biochem. Z.* **32**, 178.

# CLX. THE CARBOHYDRATE METABOLISM OF CERTAIN PATHOLOGICAL OVERGROWTHS.

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AN important biochemical aspect of the cancer problem has been stressed by Warburg and his collaborators (1924-1927) in their study of the carbohydrate metabolism of surviving tissues, normal and pathological.

An enquiry into the problem whether the basic chemical reactions which yield energy for the maintenance and growth of cells are the same in kind and velocity for normal and abnormal tissues has resulted in establishing the fact that these tissues exhibit fundamental differences in their manner of utilising carbohydrates.

Warburg has shown that malignant tissues, like certain varieties of yeasts, possess the dual capacity to metabolise carbohydrates by oxidative and splitting processes.

Normal growing tissues, while using the oxidative process (respiration) almost exclusively under *aerobic* conditions, have the power of splitting carbohydrate to lactic acid under *anaerobic* conditions (glycolysis).

The characteristic property of the cancer cell is its power and habit, even under *aerobic* conditions, to metabolise carbohydrate by both these processes.

That the energy freed by the glycolytic process (amounting, in the case of tumour tissue to about 35-40 % of that freed by respiration) is utilised in the cell economy, cannot be definitely established, but experiments on survival of tumour cells *in vitro* by Okamoto [1925] suggest that this is the case, while Warburg's studies on the inhibiting effect of narcotics on the glycolytic functioning of tumour cells would seem to show that glycolysis is a reaction associated with cell structures, the liberated energy being at the disposal of the cell.

In this connection the following questions arise. (1) Is this peculiarity of metabolism an exclusive feature of malignant tissue? (2) Are there quantitative relationships between the magnitudes of the respiration and the *aerobic* and *anaerobic* glycolyses, which afford a means of differentiating normal from pathological tissues?

Warburg has, at various times, suggested the following generalisations.

(a) The ratio  $\frac{\text{aerobic glycolysis}}{\text{respiration}}$ : this was found to be approximately three for

tumour tissue, and small or zero for normal tissues; (b) the value  $U$ , or excess fermentation, based upon the assumption that the Pasteur reaction is functioning at its maximum efficiency.  $U$  would then represent the minimum theoretical value for the aerobic glycolysis. For tumour tissue, this expression was found to be positive, for normal tissues, negative.

Many facts have been brought forward, which suggest that such attempts to differentiate malignant tissues, with respect to their carbohydrate metabolism, from normal resting or growing tissues are not fully justified.

Murphy and Hawkins [1925] found great variability in their results. Rat placenta, according to these authors, behaved as a malignant tissue, and many spontaneous mouse tumours fell into the category for normal resting tissues. Pentimalli [1927], regarding tumours as the result of a repeatedly disturbed regeneration process, found that regenerating muscle in the abdominal wall of young chicks behaved, with respect to its glycolytic metabolism of carbohydrates, qualitatively in the manner of tumour tissue, though quantitatively the results were considerably smaller. Fleischmann and Kubowitz [1927] found that the leucocytes of geese show a carbohydrate metabolism similar to that of tumours. Warburg [1927] records the fact that red blood corpuscles also possess a positive  $U$  value, and suggests that this anomaly may be due (as also with white blood cells) to the fact that they are free-living cells which are nearing the end of their life-cycle in the circulation. Warburg, Krebs [1927] and Tamiya [1927] have found that the retina is an exceptional tissue, since it possesses a glycolytic capacity of greater magnitude than that of any other animal tissue. In this case Warburg has introduced a new conception, showing acceleration or retardation of anaerobic glycolysis with the progressive development of the tissue, to differentiate tumour from retinal metabolism.

For normal tissues the value  $\frac{dQ_M^{N_1}}{dt}$  (see p. 1291) is negative, for tumour tissues zero, and for retina tissues positive.

It appeared of interest, in the light of extrinsic hypotheses on the cause of carcinogenesis, to study the carbohydrate metabolism of overgrowths associated with recognised viruses, and accepted generally as being devoid of the essentials of malignancy.

The lesions of fowl pox in pigeons, vaccinia lesions in young chickens, vaccinia lesions in rabbits, and human warts have been examined up to the present time. A study of the metabolism of brain tissue of cavies dying of rabies has also been made, where an intracellular virus is active, but unaccompanied by any tissue proliferation. The Rous sarcoma has also been examined.

Where proliferation has taken place and a gross lesion is manifested, the carbohydrate metabolism was found to be greatly enhanced, and in a manner analogous to that of tumours. Where no hyperplasia was present, no deviation was found from the normal values for the tissue concerned.

## TECHNIQUE AND EXPRESSION OF RESULTS.

The manometric technique elaborated by Warburg has been followed throughout. It is fully described in Warburg's collected papers [1926]. Any variations from the standard methods of determination of the metabolism quotients are referred to under separate headings.

The mode of expressing results is that used by Warburg. Simultaneous measurements are made of respiration, aerobic and anaerobic glycolysis, and their magnitudes expressed as the number of mm.<sup>3</sup> of gas consumed or evolved per mg. of dried tissue per hour.

$$\begin{aligned}
 Q_{O_2} \text{ (respiration)} &= \frac{\text{mm.}^3 \text{ oxygen consumed}}{\text{hours} \times \text{mg. dry tissue}} \\
 Q_M^{O_2} \text{ (aerobic glycolysis)} &= \frac{\text{mm.}^3 \text{ CO}_2 \text{ evolved by lactic acid formation in O}_2}{\text{hours} \times \text{mg. dry tissue}} \\
 Q_M^{N_2} \text{ (anaerobic glycolysis)} &= \frac{\text{mm.}^3 \text{ CO}_2 \text{ evolved by lactic acid formation in N}_2}{\text{hours} \times \text{mg. dry tissue}}
 \end{aligned}$$

## FOWL-POX.

In measuring the metabolism quotients of these lesions, certain inherent difficulties arise, which are not met with in the more straightforward cases of transplanted animal tumours or normal animal tissues. In order to get the maximum effects *in vitro* of dissolved gaseous and solid metabolites, perfect diffusion of these to the cells most remote from the surface must be ensured. Under the experimental conditions used, it can be calculated theoretically that the maximum thickness of the tissue slices must be no greater than 0.5 mm. Owing to the fatty nature of these lesions, the practical difficulties of obtaining such thin sections are great, with the result that the quotients obtained are probably less than the true values which would be found under perfect conditions of diffusion. These lesions moreover are not homogeneous in character, and allowances must obviously be made on this account.

The sections were prepared by cutting perpendicularly to the skin surface, and, on the average, each section had an area of approximately 0.5 cm.<sup>2</sup>, and a dry weight of 15–30 mg. A histological examination showed a greatly thickened and proliferated epidermal layer, a high percentage of fat, connective tissue, and occasionally a few muscle cells.

The muscle cells, as shown by Meyerhof [1921], Pentimalli [1927], and controls quoted later for Rous tumours, show a very low carbohydrate metabolism under the experimental conditions. Figures quoted by Warburg [1926] indicate that connective tissue shows a negligible metabolism, and it seems probable that the fat makes no contribution to the results obtained.

On the basis of both histological and chemical estimations of fat, a minimum of 50 % of the total dry weight was found. It seems justifiable, therefore, to multiply the actual figures obtained by at least 2, in calculating the metabolism of the proliferating epithelium. The following table of results shows the experimentally obtained quotients for a series of lesions at their maximum phase of hyperplasia.

Table I. *Carbohydrate metabolism quotients of fowl-pox lesions.*

No. of days after transmission	$Q_{O_2}$	$Q_{O_2}^M$	$Q_{N_2}^M$	Meyerhof quotient	$U$
10	-3.3	+4.7	+10.0	1.6	+3.4
9	-3.8	+5.1	+11.8	1.7	+4.2
10	-4.4	+6.5	+12.3	1.3	+3.5
9	-4.8	+5.2	+8.4	0.7	-1.2
9	-1.1	+3.3	+10.0	—	+7.8
7	-6.0	+5.2	+8.5	0.6	-3.5
5	-3.2	+5.5	+11.3	1.8	+4.9
8	-4.3	+5.9	+12.4	1.5	+3.8
13	-3.1	+2.8	+8.3	1.8	+2.1
11	-7.8	+8.5	+18.0	1.2	+2.4
15	-2.1	+2.9	+7.3	2.1	+3.1

The measurements were made in Ringer solution, containing glucose and sodium bicarbonate, at 37.8°, the gas phase being 5 %  $CO_2$  in oxygen for aerobic, and 5 %  $CO_2$  in nitrogen for anaerobic measurements.

The composition of the saline medium used was:

Salt	Moles per litre
NaCl	0.121
KCl	0.0025
$CaCl_2$	0.0018
$NaHCO_3$	0.0025
Glucose	0.2

In dealing with sections composed of different types of tissue, it is clear that the aerobic values obtained (dependent upon a calculation from the observed gas-exchange in two vessels containing different amounts of nutrient media) would only be valid if the sections used were entirely similar in composition. This condition is difficult to obtain, and two methods have been used to minimise errors arising from this:

- measurements were made with adjacent sections;
- measurements were made with the same section, making consecutive observations in different volumes of Ringer solution.

The results obtained have been similar by both methods. These results, making a minimum allowance of 50 % for the fat included in this dry weight, may be summarised as follows:

$Q_{O_2}$	$Q_{O_2}^M$	$Q_{N_2}^M$	M.q.	$U$
-6.6 to -15.6	+9.4 to +17.0	+20 to +36	1.2 to -2.1	+4.8 to +15.6

A comparison of these values with those obtained by Warburg for animal, human and avian tumours, shows them to be of the same order of magnitude, and to possess the same relationships to one another. In particular, the excess fermentation,  $U$ , is positive in nearly every case, making these lesions fall into the category of malignant tumours, if the most recent classification of Warburg be adopted.

Certain results obtained with embryonic tissue and liver show different values for the aerobic glycolysis when measured in serum from those obtained

by measurements in Ringer. Warburg attributes this to the extreme sensitiveness of the Pasteur reaction, the respiratory activity failing to function effectively owing to rapid damaging of tissue placed in Ringer solution. Hence the metabolism of fowl-pox lesions was measured in serum, both foreign (inactivated horse-serum) and autologous serum (pigeon).

Table II gives the results of a series of experiments, and indicates that the change of medium has little effect on the metabolism quotients.

Table II.

Serum	No. of days after transmission	$Q_{O_2}$	$Q_{O_2}^M$	$Q_M^{N_2}$	M.q.	$U$
Pigeon	4	-1.9	+ 4.8	+ 7.0	1.2	+ 3.2
	10	-4.6	+ 12.9	+ 20.5	1.65	+ 11.3
	11	-2.1	+ 9.1	+ 16.3	3.0	+ 12.1
	10	-4.2	+ 13.0	+ 20.5	1.8	+ 12.1
	13	-1.0	+ 2.7	+ 7.5	—	+ 5.5
	(regressing)					
Inactivated horse	7	-4.0	+ 3.3	+ 8.2	1.2	+ 0.2
	6	-3.4	+ 2.8	+ 7.3	1.3	+ 0.5
	9	-3.1	+ 5.8	+ 9.6	1.2	+ 3.4
	10	-4.2	+ 5.9	+ 11.3	1.3	+ 2.9

In order to see if there was a correlation between the metabolism and the progressive epithelial hyperplasia, a series of measurements was made from day to day of the developing lesions. The results are represented graphically in Fig. 1.

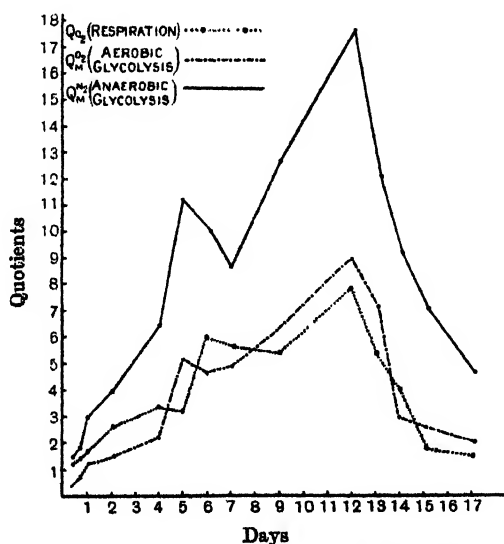


Fig. 1. Curves showing the changes in the carbohydrate metabolism of fowl-pox lesions, during development and regression.

Normal pigeon skin gave low values for all the quotients,

$$\text{e.g. } Q_{O_2} = -0.9; Q_M^{O_2} = +0.5; Q_M^{N_2} = +1.7.$$

As the lesion attained its maximum growth (12 or 13 days), the respiration, aerobic and anaerobic glycolysis increased, and with its slow regression all these quotients decreased to the level typical of normal skin.

To account for the aerobic glycolysis found in red and white blood cells, Warburg suggests that they are in a degenerating condition. The progressive character of the metabolism with proliferation of the epithelium in fowl-pox lesions, where histological examination shows numerous mitotic figures, suggests that the concept of degeneration does not account for such increases as are observed. Moreover, when regression commences, and it would be expected that degeneration would follow, the glycolytic metabolism tends to disappear.

### VACCINIA.

(a) *Rabbit*. The vaccinia lesion consists of dense infiltration of the dermis with polymorph leucocytes. The adventitial or reticulo-endothelial cells are numerous and in active mitosis. There is no marked change in the Malpighian layer, except early chromatolysis and swelling. Mitotic figures are occasionally seen.

Table III shows the quotients obtained in two experiments with these lesions, and with normal rabbit skin.

Table III.

Tissue	$Q_{O_2}$	$Q_M^{O_2}$	$Q_M^{N_2}$
Vaccinia lesion (rabbit)	-3.3	+2.3	+2.8
	-3.0	+2.0	+2.7
	-1.0	+1.3	+1.9

Sections perpendicular to the skin surface were used and the measurements made in Ringer solution containing glucose and bicarbonate. The figures refer to the whole lesion, with no allowances for material of very small, or no, measurable metabolism.

The increase in metabolic activity is small, and might be attributed to the presence of leucocytes.

(b) *Young chickens*. The lesions produced in one to seven days old chickens by the vaccinia virus are identical histologically with those produced by the fowl-pox virus, as shown by Findlay [1928] and Ludford [1928].

Table IV.

Tissue	$Q_{O_2}$	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	$U$
Vaccinia lesion	-5.0	+4.2	+11.1	1.4	+1.1
	-3.2	+3.6	+7.8	1.3	+1.4
	-4.8	+4.0	+10.6	1.4	+1.0
Normal chicken skin	-1.0	+0.4	+1.5	—	—

Corresponding to the epithelial hyperplasia, a great increase in the carbohydrate metabolism is noticed (Table IV).

The measurements were made in Ringer solution, and the figures represent the metabolism of the whole lesion. The same difficulties of obtaining sections of the necessary thickness for perfect diffusion of metabolites and the presence of non-reacting tissues make the values quoted minimal.

#### HUMAN WARTS.

The metabolism quotients found in the three cases examined are much smaller than those found for fowl-pox lesions (Table V).

The gas exchanges observed during the experiments were comparatively large, and suggest an active metabolism for the relatively small percentage of physiologically active epithelial cells present. Considerably more than half of the section was composed of fully keratinised epithelium and connective tissue, giving the dry weight a value many times greater than that of the reacting portion.

Table V.

	$Q_{O_2}$	$Q_M^{O_2}$	$Q_M^{N_2}$	M.q.	$U$
A	-1.7	+2.2	+4.1	1.1	+0.7
B	-1.2	+1.7	+3.9	1.8	+1.5
C	-1.2	+1.6	+3.8	1.8	+1.4

#### RABIES.

The strain of virus used was a "virus fixe" which is highly pathogenic for guinea-pigs, and on intracerebral inoculation produces a fatal result in a week. The animals were killed when moribund and the brain removed for examination.

Sections were cut tangentially from the cerebral cortex and hippocampal region.

The average quotients obtained for the outer layer of normal guinea-pig cerebral cortex were:

$$Q_{O_2} = -10.3; Q_M = +2.5; Q_M^{N_2} = +20.3.$$

Table VI shows the figures obtained for the brain of guinea-pigs dying of rabies.

Table VI.

$Q_{O_2}$	$Q_M^{O_2}$	$Q_M^{N_2}$
- 9.2	+2.9	+25.6
- 8.8	+2.7	+18.8
- 7.0	+1.0	+20.4
-10.8	+1.9	+20.2

Here no hyperplasia is found and the activities of an intracellular virus seem to cause no alteration in the carbohydrate metabolism of the cells.



## ROUS CHICKEN SARCOMA.

Warburg only quotes one experiment on the metabolism of this tissue, showing a behaviour similar to that of the rat tumours, studied so extensively.

Many measurements, in Ringer solution, have been made and Table VII shows the great variability in the results obtained. The respiration was, in some cases, found to be abnormally high, which had the effect of making  $U$  negative, and the Meyerhof quotient very low. In all cases a histological control showed no areas of necrosis, only the outer shell of the growth being used for the experiments. It has not been possible to correlate these wide variations with the age of the bird or the general necrotic or haemorrhagic conditions of the tumour.

Table VII.

No. of days after transmission with cell-free filtrate	$Q_{O_2}$	$Q_{O_2}^M$	$Q_M^{N_2}$	M.q.	$U$
10	- 8.7	+20.3	+27	0.8	+ 9.6
11	- 4.0	+13.8	+21.8	2.0	+13.8
13	-22.0	+23.9	+36.6	0.6	- 7.4
14	-26.9	+21.3	+30.1	0.3	-23.7
14	-10.1	+18.1	+28.1	1.0	+ 7.9
10	-20.0	+21.2	+33.3	0.6	- 6.7
10	-10.4	+16.3	+31.8	1.5	+11.0
11	- 7.2	+16.2	+29.2	1.8	+14.8
12	- 4.8	+22.6	+33.8	2.3	+24.2
13	- 7.2	+21.8	+35.5	1.9	+21.1

In order to study any possible immediate effect of the agent responsible for the initiation of Rous sarcoma on the metabolism of the muscle cells near the site of injection, a series of measurements was made at daily intervals from the time of transmission.

Cut muscle under the experimental conditions shows a low metabolism and a control experiment was made simultaneously with muscle from the opposite breast of the bird. A typical value obtained for cut muscle is:

$$Q_{O_2} = -2.5; Q_M^{O_2} = +1.2; Q_M^{N_2} = +2.5.$$

A slight increase in metabolic activity of the muscle was noticed during the first two days after injection of cell-free filtrate (possibly due to leucocytic reaction) followed by a return to the normal low values of the undisturbed muscle. Only when a histological examination showed foci of tumour cells scattered through the muscle was any real increase found in the metabolism quotients. The figures obtained showed a rough correspondence in their magnitude to what might be theoretically calculated from the proportion of tumour tissue seen microscopically. It would seem correct to conclude that the tumour cells possess their characteristically high metabolic rate from the time of their appearance rather than that a progressive development of this metabolism takes place over a transitional period.

SUMMARY AND DISCUSSION.

1. Using the technique developed by Warburg, the carbohydrate metabolism of a series of lesions associated with intracellular viruses has been examined.

2. The activities of the virus in fowl-pox lesions, vaccinia lesions in young chickens, and in human warts, are accompanied by epithelial hyperplasia and in these cases an active metabolism has been found, corresponding in type to that characteristic of malignant tissue.

Making allowances for non-reacting tissue in the sections examined, the magnitude of the respiration and aerobic and anaerobic glycolysis approximates to that found for tumours.

3. In the case of vaccinia lesions in rabbits, where little or no epithelial hyperplasia is evident, the lower quotients obtained are probably due to leucocytic invasion.

4. The brain of guinea-pigs dying of rabies provides an example of a tissue where a virus is active without stimulating the cells to abnormal division, and no deviation is found from the normal metabolism.

5. The metabolic activity of fowl-pox lesions shown graphically exhibits a rough parallelism in its magnitude to the state of development or regression of the lesion.

6. Great variability in the values for respiration in the Rous chicken sarcoma are recorded.

7. After the injection of Rous sarcoma cell-free filtrate in the muscle of fowls a slight rise in the metabolism was noticed during  $4\frac{1}{2}$  hours, followed by a return to the normal values obtained for resting muscle.

During the subsequent development of the tumour in its early stages, the figures obtained indicate that Rous sarcoma cells, on their first appearance, assume the high metabolic activity characteristic of the fully grown tumour.

The general conclusion which may be drawn from these results would seem to be that the magnitude and relationships of the respiratory and glycolytic processes, found by Warburg to be characteristic of malignant tissues, are not specific for malignant tissues but are a common feature of pathological overgrowths.

The author's thanks are due to Prof. Warburg of Berlin-Dahlem for demonstrating his methods, and to Dr G. Marshall Findlay of the Imperial Cancer Research Fund for his ready help in obtaining material and conducting the animal experiments.

## REFERENCES.

- Findlay (1928). *Proc. Roy. Soc. Lond. B*, **102**, 354.  
Fleischmann and Kubowitz (1927). *Biochem. Z.* **181**, 395.  
Krebs (1927). *Biochem. Z.* **189**, 57.  
Ludford (1928). *Proc. Roy. Soc. Lond. B*, **102**, 406.  
Meyerhof (1921). *Pflüger's Arch.* **188**, 114.  
Murphy and Hawkins (1925). *J. Gen. Physiol.* **8**, 115.  
Okamoto (1925). *Biochem. Z.* **160**, 52.  
Pentimalli (1927). *Z. Krebsforsch.* **25**, 347.  
Tamiya (1927). *Biochem. Z.* **189**, 114.  
Warburg (1926). *Über den Stoffwechsel der Tumoren* (Springer, Berlin).  
Warburg (1927). *Biochem. Z.* **184**, 484.

# CLXI. THE PURINOLYTIC ENZYMES OF THE LEECH (*HIRUDO MEDICINALIS*) AND THE FRESH-WATER MUSSEL (*ANODONTA*).

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*(Received August 13th, 1928.)*

## INTRODUCTION.

URIC acid is in almost every case a stage or else the final product of purine metabolism within the living organism. Certain cases have, however, been reported where this stage appears not to enter. Thus Ulpiani and Cingaloni [1906] found that certain bacteria derived from poultry excreta degrade aminopurines in a different manner, involving the formation of guanidine; in this case, therefore, neither uric acid nor allantoin can be amongst the products of purinolysis.

Certain invertebrate forms are known [Przylecki, 1926], *e.g.* leeches and fresh-water mussels, amongst the final products of whose purine metabolism uric acid or allantoin cannot be found. In the case of leeches uric acid added to their tissues is not oxidised, whilst *Anodonta* is able quantitatively to destroy added uric acid. Two explanations of these phenomena might be given. One would be that the purine catabolism of these animals runs a course similar to that of Ulpiani and Cingaloni's bacteria, whilst the other would be that purine catabolism stops short at some stage prior to the appearance of uric acid. The first supposition would involve a fall in the purine-N content of the tissues after autolysis of over 20 %; in the second case the loss of purine-N due to autolysis should not exceed 20 % if both guanase and adenase are present, or some smaller figure if only one of these enzymes exists, and would be nil if neither of these ferments is possessed by these animals.

## EXPERIMENTAL.

**A. Leeches.** These animals were first placed in concentrated sodium chloride solution, with the object of forcing them to disgorge any blood present in the alimentary canal, and were then washed, roughly dried with filter paper, and comminuted using either scissors or a mincing machine. Four portions of 50 g. each of the well-mixed mince were weighed out and placed in litre flasks, to which 500 cc. of water were added. Toluene was added to two flasks, which were left in a thermostat at 37° for about two weeks. Sulphuric acid to 5 %

was then added and the contents of the flasks were boiled under reflux for 4 hours, after which total- and purine-N were determined by the method of Krüger and Schittenhelm, as described in a previous paper [Truszkowski, 1926]. A portion of mince was also weighed out for the determination of dry content, being dried at 110° to constant weight. The contents of the remaining two flasks were hydrolysed immediately. The results are given in Table I, each value being the mean of two determinations. The dry content varies from 19.25 to 27.2 %, average 22.5 %, and the total-N content from 2.718 to

Table I. *Total- and purine-N contents of leeches and fresh-water mussels before and after autolysis.*

Material No.	% solid substance	% N live wt	% N dry wt	Before autolysis		Days autolysis	After autolysis		% diminution in purine N
				Purine-N mg. per 100 g. live wt	Purine-N mg. per 100 g. dry wt		Purine-N mg. per 100 g. live wt	Purine-N mg. per 100 g. dry wt	
Leeches 1	21.15	2.718	12.85	68.4	323.6	17	63.0	280.2	7.9
2	19.25	2.344	12.18	48.0	249.1	15	36.3	189.2	24.4
3	27.2	3.675	13.53	53.8	197.7	16	44.2	161.8	17.8
Mean	22.5	2.912	12.85	56.7	256.8	—	47.8	210.4	16.7
Mussels 1	12.7	1.179	9.27	36.2	284.8	—	—	—	—
2	13.0	1.093	8.41	34.0	261.5	15	28.1	216.5	17.4
3	12.75	0.963	7.55	36.0	282.6	16	31.8	249.5	11.7
4	11.1	1.001	9.10	34.6	314.3	13	29.8	270.8	13.9
Mean	12.39	1.059	8.58	35.2	285.8	—	29.9	245.6	14.3

3.675 %, average 2.912 %. Considerable variations are found in purine-N, from 48.0 to 68.4 mg. per 100 g., mean 56.7 mg. per 100 g. After autolysis (15–17 days) the mean purine-N content falls to 47.8 mg. per 100 g. giving a mean loss of 16.7 %. This indicates deamination of both aminopurines present. The absence of uniformity of the results obtained is probably due to the incomplete elimination of blood from the alimentary tract.

B. *Fresh-water mussels*. These were taken from clay-pits in the vicinity of Warsaw at various seasons of the year, from spring to autumn; all belonged to various sub-types of the genus *Anodonta*. After separation from the shell, they were allowed to drain for a few minutes, and passed through a mincing machine. The well-mixed mince was then treated as above for the determination of dry content, total- and purine-N. In the determination of purine-N, however, it was found that Krüger and Schittenhelm's method was not, without certain modifications, applicable. This was due to the extraordinarily high calcium content of these animals; thus in No. 2, the filtrate after acid hydrolysis contained 1.4 g. of calcium oxide, amounting to over 21 % of the dry weight of tissue taken and the residue from hydrolysis contained a further quantity of insoluble calcium sulphate. This calcium, whilst soluble in strongly acid solution, begins to separate as a flocculent precipitate at about  $p_H$  4, precipitation being complete at  $p_H$  8, at which  $p_H$  sodium hydrogen sulphite

and copper sulphate are added for the precipitation of purines, and accompanies the purines through all the subsequent stages of the determination. Its harmful influence is particularly noticeable in the dissolution of the purine-copper double salt by means of sodium hydrosulphide, when, even after adding comparatively very large quantities of the sulphide, copper is continually found in the filtrate. This effect appears to be due to preferential formation of calcium sulphide or hydrosulphide.

This source of error was eliminated by the addition of excess ammonium oxalate to the hydrolysate, after its neutralisation and feeble acidification with acetic acid.

The dry content of *Anodonta* (Table I) varies from 11.1 to 13.0 %, mean value 12.4 %. The nitrogen content varies from 0.963 to 1.179 %, mean value 1.059 %, and the purine-N content from 34 to 36 mg. per 100 g., mean 35.2 mg. per 100 g. After autolysis (13-16) days the mean purine-N content fell to 29.9 mg. per 100 g., a decrease of 14.3 %. It appears therefore that in this case, too, both guanase and adenase are present.

#### DISCUSSION.

Since both leeches and fresh-water mussels are capable of deaminising guanine and adenine, but do not produce uric acid, it follows that in these animals, purine catabolism stops at some stage short of uric acid. That these animals do not degrade purines in some other way not involving deamination and the production of uric acid is indicated by the limited loss of purine-N during autolysis.

It hence follows that the end-product of purine catabolism of leeches and *Anodonta* must be xanthine and/or hypoxanthine. Przylecki's classification [1926] of leeches as uricostatic-uriconegative forms and of *Anodonta* as uricolytic-uriconegative forms is thus confirmed.

#### SUMMARY.

1. Purine-N has been determined before and after autolysis of the tissues of leeches and of fresh-water mussels.

2. The results obtained indicate that the end-product of their purine catabolism is xanthine and/or hypoxanthine.

This research was undertaken at the suggestion of Prof. S. J. Przylecki, to whom I wish to express my sincere gratitude for his help and advice.

#### REFERENCES.

- Przylecki (1926). *Arch. int. Physiol.* **27**, 197.  
 Truszkowski (1926). *Biochem. J.* **20**, 437.  
 Ulpiani and Cingaloni (1906). *Atti R. Accad. Lincei*, **14**, II, 596.

## CLXII. STRUCTURE AND ENZYME REACTIONS.

### PART VII. THE SYSTEM GLYCOGEN-AMYLASE-LIVER TISSUE.

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THE researches described in the previous parts of this series [Przylecki *et al.*, 1927, 1928; Truszkowski, 1928], although performed upon models under conditions differing greatly from those obtaining within the living cell, yielded results possessing a certain biological importance, notably in the elucidation of the rôle of structure in the attainment of definite intracellular equilibrium.

It has been shown that the introduction of certain natural cell-constituents into the system amylase-glycogen leads to a retardation of the velocity of the reaction, due to the adsorption of either the ferment, the substrate, or both. We have, further, found that amylase becomes inactivated by adsorption upon a lipoid mixture, whilst adsorption upon protein in no way impairs its activity. It appears, therefore, that the velocity of glycogenolysis in the presence of these cell-constituents depends not only upon that of the ferment.

One of the problems whose solution was sought in this series of researches was that of the domination within the cell of reactions of synthesis over those of hydrolysis. On the basis of the experiments described in Parts v and vi of this series it was concluded that, in view of the strong adsorption of glycogen upon cell-proteins, and of the feeble adsorption of glucose, the reaction  $\text{glucose} \rightleftharpoons \text{polysaccharides}$  should be predominantly one of synthesis in a cellular environment. The conditions under which the experiments on which this conclusion was based are, however, not entirely comparable with those obtaining within the cell.

It was therefore necessary to study this problem under conditions closer to those actually found within the cell, not only in so far as concerns the nature of the adsorbent itself, but also in order to establish whether changes in the equilibrium point in favour of the reaction of synthesis of glycogen are due exclusively to adsorption of both constituents, or whether some further factors come into play, as indeed appeared to us to be probable.

In view of the high degree of adsorption of amylase found by us, and of the efficient action of such adsorbed amylase, the equilibrium point of the reaction

glucose  $\rightleftharpoons$  glycogen, in the system glycogen-glucose-amylase-protein is, as we have shown, dependent upon the ratio of unadsorbed glycogen to glucose. The degree of glycogenolysis depends, therefore, upon the reversibility of the adsorption of glycogen.

The first question to be settled was whether glycogen in adsorption upon protein can be eluted by addition of water, and experiment showed that this was the case. In view of this, equilibrium in this system would depend upon the two equilibrium relations:

adsorbed glycogen  $\rightleftharpoons$  free glycogen (I);

free glycogen  $\rightleftharpoons$  glucose (II).

The elution of glycogen from protein would proceed until definite equilibrium had been attained in the system free glycogen-glucose, and this equilibrium again would depend upon the ratio adsorbed glycogen : free glycogen. Thus the greater the percentage of glycogen in adsorption, the sooner would equilibrium II supervene, and the quantity of adsorbed glycogen would be greater, the greater the ratio of free glycogen to glucose. In view of the above, we should expect to obtain minimum glycogenolysis in the presence of active amylase under the following conditions.

1. In order to bring the ratio of adsorbed to free glycogen to a maximum, very large quantities of protein together with small concentrations of glycogen should be taken; that is, however, inconsistent with biological conditions.

2. In order to attain an optimum ratio of free glycogen to glucose, solutions containing high concentrations of glucose and colloids should be taken. The latter point appeared to us to be of special importance.

The first question examined experimentally therefore was whether the deposition of glycogen in liver cells could be explained as being due exclusively, or to a large extent, to adsorption on the surfaces of the various hepatic colloids. This appeared to us to be *a priori* somewhat improbable, but, nevertheless adsorption isotherms of glycogen on liver pulp were constructed. The liver pulp used was left for a considerable period of time with antiseptics, which were then removed, and the pulp was thoroughly washed. The product contained no amylase or polysaccharides, and was also partly freed from lipoid substances. The reversibility of adsorption of glycogen on this pulp was next studied, and finally, having settled this point, the equilibrium point of the reaction glycogen  $\rightleftharpoons$  glucose was determined in the system adsorbed amylase-glycogen-liver protein, using large quantities of the last-named substance.

## EXPERIMENTAL.

### I. Adsorption.

The degree of adsorption on liver pulp of glycogen from solutions of various concentrations was determined according to the methods described in Part III of this series [1928]. The liver pulp was prepared by adding to 1 kg. of comminuted, and, as far as possible, blood-free liver, 100 cc. of chloroform, 200 cc.



of glycerol and 1 litre of water. The whole was left for some months, during which time amylase and glycogen passed into solution, and the latter underwent hydrolysis to glucose. The contents of the flask were then poured through a sieve, on which the residue was washed in a strong current of water for 48 hours. The resulting product, which was free from amylase and polysaccharides, was allowed to drain, and was then used as the adsorbent. To 10, 20, or 30 g. portions of this pulp 30 cc. of 0.5–10 % glycogen solution were added, and the whole was shaken for 4 hours. The mixture was then either filtered, or centrifuged at low velocities in order to avoid concentration of glycogen in the lower layers. Glycogen was determined either in the filtrate or in the pulp, using the Pflüger-Bertrand method.

Table I. *Adsorption of glycogen on liver pulp.*

In every case 30 cc. of glycogen solution were used, and the pulp was rubbed through a sieve before weighing out.

Glycogen solution used		Adsorption of glycogen on											
		10 g. pulp (analysis of pulp)		20 g. pulp (analysis of solution)						30 g. pulp (analysis of pulp)			
				Exp. 1			Exp. 2						
				Free gly- cogen in solution			Free gly- cogen in solution						
%	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.	%	mg.
0.5	150	53	79.5	96	144	0.018	82	123	0.088	—	—	—	—
1.0	300	52	156	94	282	0.054	85	253	0.179	99.0	297	0.099	—
2.0	600	46	276	66.6	400	0.60	79	474	0.416	—	—	—	—
3.0	900	43	387	68.8	619	0.57	70	630	0.891	93.0	837	0.21	—
5.0	1500	40	600	—	—	—	72	1080	1.39	—	—	—	—
7.5	2250	—	—	76.0	1710	1.62	—	—	—	—	—	—	—
10.0	3000	—	—	78.1	2343	1.97	74	2220	2.57	89.1	2673	1.08	—

The results, given in Table I, show that the pulp has an extraordinarily high adsorptive power for glycogen, and that, using appropriate concentrations of pulp, corresponding to those present in the liver, this adsorption may, even for 10 % glycogen solutions, amount to as much as 90 %. This fact appears to be of great interest, as it indicates that, within the cell, glycogen is concentrated on the protein surfaces. These results are, moreover, in conflict with certain histological evidence, whereby it would appear that glycogen is concentrated on lipid surfaces. We cannot yet say whether the inability to adsorb glycogen found by Truszkowski for certain lipoids applies to all lipoids, or whether the drops of lipid substances within the cell are coated with some protein, on the surface of which glycogen may be adsorbed. It is also possible that diminution of the surface of drops of lipoids due to several drops coalescing into one large one may lead to the irreversible precipitation of glycogen or starch adsorbed on these surfaces. This question will be considered in a later paper.

It follows from the results described in this paper that, where a cell system contains up to 10 % of glycogen, barely 0.8 % or even less can exist in solution, the remainder being adsorbed upon protein and possibly lipid surfaces.

## II. *Reversibility of adsorption.*

Three points of biological significance were here to be elucidated. The first was to establish with what facility glycogen undergoes elution from protein as a result of dilution. The second was to determine the extent of adsorption of glycogen from solutions of various concentrations. Finally, the percentage of glycogen remaining in solution after the addition of different volumes of the same glycogen solution to equal quantities of adsorbent was measured.

The preliminary experiments were carried out on liver pulp or coagulated egg-white on which glycogen was adsorbed. Analysis of the adsorbent after washing in a stream of water for 24–48 hours showed that barely traces of glycogen remained. It hence follows that glycogen can be quantitatively eluted from adsorption on protein without the agency of elutive factors other than water. Further experiments were of a quantitative nature, the percentage elution at given dilutions being determined. The results are given in Table II.

Table II. *Elution by dilution.*

In every case 20 g. of liver pulp were taken.

In Exp. 4 30 cc. of 5 % glycogen were added to 20 g. of pulp, the whole was shaken for 4 hours, left for 3 hours, 270 cc. water were then added, the whole was shaken a further 6 hours and left 12 hours, after which glycogen was determined.

No. of Exp.	Glycogen g.	Water cc.	Glycogen %	Adsorbed glycogen		Glycogen in solution	
				g.	%	g.	%
1	0.150	30	0.5	0.123	82	0.027	0.088
2	1.500	30	5.0	1.170	78	0.330	1.09
3	1.500	300	0.5	0.688	46.5	0.812	0.27
4	1.500	300	0.5	0.741	49.4	0.759	0.25

## III. *Definitive equilibrium.*

The following experiments have only a theoretical significance, their object being to establish whether in the presence of an adsorbent, definitive equilibrium supervenes before the hydrolysis of all the glycogen present in a system containing on the average 70–80 % of water.

These experiments were performed on solutions of glycogen, the adsorbent being coagulated egg-white, on which amylase had been adsorbed. 10 g. of egg-white with adsorbed amylase were taken, and 5 cc. of solutions or suspensions of 1 to 10 % glycogen. These systems were kept under aseptic conditions, without the introduction of antiseptics. In order to favour the reaction  $\text{glucose} \rightarrow \text{glycogen}$ , glucose was added to 1–2 % of the reaction solution. The glycogen content of the whole system was determined after 1, 2 and 4 weeks. The results indicated that glycogenolysis proceeds practically to completion in these circumstances.

As to the cell system, the above experiments indicate that adsorption is able to inhibit glycogenolysis and to keep glycogen stabilised for an indefinitely long period only when glycogen is present exclusively in adsorption, or when

the reaction glycogen  $\rightleftharpoons$  glucose is close to equilibrium, so that it is able to proceed in one direction or another when changes supervene in the concentration of one or the other substrate. Only in these circumstances can the synthesis of polysaccharides take place.

For the realisation of this synthesis not only the presence of an adsorbent which would remove large quantities of glycogen from solution is necessary, but, further, conditions should be such that the reaction glycogen  $\rightleftharpoons$  glucose should be easily reversible. Such conditions cannot be brought about by the presence of an adsorbent. For this purpose, not only should the relative concentration of glycogen be diminished but also that of water. This matter will be considered more fully in a subsequent communication.

A further conclusion may be drawn from the above results. This is that our hypothesis is confirmed that the velocity of reaction is, in view of the traces of glycogen left in solution, determined in the first place by the equilibrium adsorbed glycogen  $\rightleftharpoons$  free glycogen, even where the concentration of polysaccharide is high.

#### SUMMARY.

1. Liver tissue has an exceptionally high adsorptive action upon glycogen.
2. Adsorption of glycogen upon tissue and other proteins is of a reversible nature, elution taking place when the concentration of glycogen in solution falls.
3. The addition of protein to the system glycogen-amylase retards but does not inhibit the reaction of glycogenolysis.
4. Biologically, glycogenolysis is the resultant of the reactions: adsorbed glycogen  $\rightleftharpoons$  free glycogen  $\rightleftharpoons$  glucose.

#### REFERENCES.

- Przylecki, Niedzwiecka and Majewski (1927). *Biochem. J.* **21**, 1025.  
Przylecki and Niedzwiecka (1928). *Biochem. J.* **22**, 34.  
Przylecki, Giedroyć and Sym (1928). *Biochem. J.* **22**, 811.  
Truszkowski (1928). *Biochem. J.* **22**, 767.

# CLXIII. DECOMPOSITION OF URIC ACID IN BLOOD.

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GAROT [1926] showed that the uric acid content of dog's blood falls considerably after digestion with "pancreatin"; this effect he ascribed to the presence of uricase in the blood of these animals. Flatow [1926] found that uric acid undergoes oxidation if added to blood, and that this process is greatly accelerated by passing air through the mixture. The uricolytic agent in this case was, according to this author, a substance which he termed "uricoid."

Up to the present, no incontrovertible evidence has been adduced for the existence of uricase in the blood of any mammal, and the nature of the substance producing uricolysis is therefore still problematical.

Biltz and Schauder [1923] and Piaux [1924] showed that a number of substances exist which catalyse the oxidation of uric acid, the most important being such metals as iron, copper, manganese. Since an iron-containing substance, haemoglobin, is present in blood, the possibility arose that this was the factor responsible for the oxidation of uric acid in blood.

Experiments will be described in this paper having as their object the elucidation of the nature of the catalyst in question and of the action of haemoglobin upon uric acid.

## EXPERIMENTAL.

### A. Determination of uricolytic agent.

Horse blood was collected at slaughter-houses, and potassium oxalate added immediately to prevent clotting. It was used generally within about 6 hours of shedding.

The uricolytic powers of whole blood, blood-plasma, erythrocytes, boiled erythrocytes, stromata and haemolysed corpuscles free from stromata were determined separately. Two types of determination were made. In one, uricolysis was determined in bottles through which a feeble stream of oxygen was passed, whilst in the other determinations were made in stoppered flasks. In all cases 50 cc. of plasma, erythrocyte suspension, etc., were taken, and 20 cc. of lithium urate solution together with 20 cc. of water were added,

with thymol as disinfectant. The reaction mixtures were placed in a thermostat at 37°, and their uric acid content determined at different intervals of time.

The solution was freed from protein by the method of Folin and Wu, 100 cc. of 10 % sodium tungstate and 100 cc. of 2/3 *N* sulphuric acid being added. Uric acid was determined in the filtrate using the colorimetric Folin-Denis method, 12 cc. of filtrate being taken for each determination. The results obtained by this colorimetric method were confirmed by determination of purine-nitrogen using the method of Krüger and Schittenhelm.

The results given in Table I show that blood-plasma possesses no uricolytic power, since even after 48 hours the uric acid content did not diminish by more than 6.8 %, and this diminution may be ascribed to adsorption. In the presence of erythrocytes uric acid undergoes oxidation. An examination of the figures obtained shows that at first adsorption takes place; during the first two hours no difference in the content of uric acid can be observed, after which it undergoes continual destruction, so that after 48 hours about 73 % is oxidised.

In order to establish whether the agent responsible for this oxidation is a catalyst or a ferment, the above experiments were repeated with erythrocytes previously heated to 100° for 10 minutes. The results (Table I) show that their uricolytic power is in no way diminished by this treatment, whence it follows that blood contains a thermostable uricolytic agent.

Whilst crude uricase is inactivated by such treatment, it has been shown by Przylecki (unpublished work) that highly purified preparations of uricase are similarly thermostable. In order to verify whether this ferment is present, the procedure employed by Przylecki was applied to the separation of uricase. The product so obtained was inactive, whence it would appear that uricase is absent from mammalian blood.

The effect upon velocity of oxidation of passing a feeble stream of oxygen through the reaction mixture was next studied. The results obtained (cf. Table I) showed a distinct acceleration of reaction, since only traces of uric acid remained after 48 hours.

We have, therefore, established that erythrocytes contain some thermostable non-enzymic substance which energetically oxidises uric acid in the presence of oxygen. The question remains whether this substance is present in the stromata or the contents of the erythrocytes. Accordingly, a suspension of haemolysed erythrocytes was centrifuged, the supernatant liquid passed through a Berkefeld filter, in order to ensure the removal of fragments of stromata, and uric acid added to the filtrate. Only traces of uric acid remained after 48 hours (Table I). This result was confirmed by determining purine-nitrogen using the method of Krüger and Schittenhelm. The purine-nitrogen content fell in 48 hours from 25.5 mg. to 4.9 mg. (see Table III). Parallel experiments on washed stromata showed only absorption of uric acid, amounting after 48 hours to 10.5 %, after which no further fall in uric acid content could be observed (Table I).

Table I.

		Original uric acid content mg. in 2 cc.	5 mins. Uric acid content mg.	30 mins. { Uric acid content mg. Loss %	1 hour and 2 hours. Uric acid content mg.	4 hours { Uric acid content mg. Loss %	24 hours { Uric acid content mg. Loss %	48 hours { Uric acid content mg. Loss %	72 hours Uric acid content mg.
Catalyst	...	...	...	...	...	...	...	...	...
Plasma	...	...	0.852 No change	0.795 6.68 0.793 6.92	No change	No change	No change	No change	No change
Mean	...	...	0.852 "	0.794 6.8	"	"	"	"	"
Fresh erythrocytes	...	...	1.165 No change	1.107 4.97 1.107 6.66	No change	1.019 12.55 1.019 14.08	0.852 26.86 0.862 27.31	0.333 71.41 0.299 75.21	
Mean	...	...	1.126 "	1.048 6.92	"	0.96 14.2	0.843 25.13	0.303 73.08	
Boiled erythrocytes	...	...	1.159 —	1.087 6.18	—	0.999 13.64	0.852 26.43	0.311 72.23	
Mean	...	...	1.165 No change	1.107 4.97 1.107 6.66	No change	1.019 12.53 1.019 14.08	0.803 31.07 0.941 20.66	0.320 71.84 0.392 69.83	
Erythrocytes with oxygen	...	...	1.126 "	1.107 1.68	"	0.862 23.52	0.666 40.9	0.284 74.8	
Mean	...	...	1.159 —	1.107 4.47	—	0.966 16.71	0.803 30.87	0.334 71.82	
Intracorporeal fluid	...	...	1.153 —	—	—	—	0.367 68.17	Traces	
Stroma 0.8 g. per portion	...	...	1.127 —	—	—	—	0.372 66.90	"	
Mean	...	...	1.14 —	—	—	—	0.369 67.58	"	
Haematin 0.5 g. per portion	...	...	0.355 —	—	—	—	—	Traces	
Mean	...	...	0.372 —	—	—	—	—	"	
	...	...	0.461 —	—	—	—	0.534 2.9	0.495 10	No change
	...	...	1.14 —	—	—	—	0.361 3.06	0.331 11.02	No change
	...	...	1.127 —	—	—	—	—	0.463 10.51	No change
	...	...	—	—	—	—	—	Traces	
	...	...	—	—	—	—	—	"	

The uricolytic agent is therefore present exclusively in the intracellular fluid, and it appears probable that it may be haemoglobin. This conclusion is further supported by experiments conducted on purified crystalline haematin, in the presence of which uric acid was similarly quantitatively oxidised.

*B. Influence of alcohols, of methylene blue and of potassium cyanide upon the oxidation of uric acid in blood.*

Butyl and propyl alcohols were added in various concentrations to solutions of haemolysed blood containing uric acid. The results, given in Table II, show that oxidation is inhibited to an extent depending upon the concentration of alcohol added.

In order to establish whether we have here to do with a reaction of true oxidation or of dehydrogenation, the influence upon the velocity of oxidation of the addition of methylene blue was investigated. The results (Table II) do not indicate any acceleration of reaction where this dye is added; on the contrary, a slight retardation is observed, possibly due to adsorption of methylene blue upon the surface of the catalyst in question.

The addition of potassium cyanide did not inhibit uricolysis (Table II).

Table II.

Substance added cc. per 100 cc. erythrocyte suspension		Original uric acid content mg./5 cc.	After 48 hours	
			Uric acid content mg./5 cc.	% loss of uric acid
Butyl alcohol	2	1.15	Traces	
		1.15	0.16	86.95
Butyl alcohol	4	1.15	0.605	47.3
Propyl alcohol	8	1.15	0.63	45.12
Propyl alcohol	16	1.15	0.785	31.73
M/500 methylene blue	10	0.372	0.145	61.02
		0.55	0.2	64.05
Mean	...	0.461	0.1725	62.76
M/1000 potassium cyanide	40	0.53	0.14	73.54
		0.64	0.16	75
Mean	...	0.58	0.15	74.27

*C. The products of oxidation of uric acid in blood.*

The following analyses were carried out with the object of identifying the products arising from the catalytic oxidation of uric acid under the influence of haemoglobin. Urea and ammonia were determined by the methods of Folin and Wu in fresh blood, and in blood to which uric acid had been added and which had remained for 48 hours at 37°. Allantoin was determined in other portions, using Wiechowski's method, and in separate portions purine-nitrogen was determined by the method of Krüger and Schittenhelm. The results, given in Table III, show that the major portion of the uric acid-nitrogen is found as urea, the remainder being ammonia-, allantoin- and purine-nitrogen.

Table III.

				Fresh erythrocytes mg. N in 100 cc.	After 48 hours mg. N in 100 cc.
Total uric acid content of blood				25.5	4.9
Ammonia	...	...	...	—	2.4
Urea	...	...	...	4.2	12.6
Allantoin	...	...	...	—	6.3
Totals	...	...	...	29.7	26.2

# DISCUSSION.

Uric acid, on standing with blood, is quantitatively oxidised within 48 hours, yielding as products of oxidation, allantoin, urea and ammonia. The catalytic agent in question is absent from the blood-plasma and from the stromata of the erythrocytes, which are able only to adsorb uric acid in quantities not exceeding 10 % of that present in solution. Haemoglobin is probably the uricolytic catalyst in question.

It is well known that iron compounds play an important rôle as oxidation catalysts within the cell [see Warburg, 1914].

Robinson [1924], who used haemoglobin and haematin as catalysts for the oxidation of linseed oil, found that haematoporphyrin was without effect, and her experiments suggest that it is the iron present in haemoglobin that is responsible for oxidation of uric acid. This author showed further that cyanides do not inhibit the catalytic action of iron present in haemoglobin. This would point to the iron being present in some non-ionised form, as is the case with that of haemoglobin.

The results obtained with methylene blue indicate that the reaction of uricolysis under the influence of haemoglobin is rather one of oxidation than one of dehydrogenation.

# SUMMARY.

1. Uric acid is quantitatively oxidised by blood within 48 hours at 37°.
2. Stromata and blood-plasma are inactive in this respect.
3. Haemoglobin is most probably the uricolytic agent present in blood.
4. The products of uricolysis in blood are allantoin, urea and ammonia.
5. The reaction of uricolysis is inhibited by propyl and butyl alcohols, but not by cyanides.
6. The reaction is one of true oxidation, and not of dehydrogenation.

The above research was undertaken at the suggestion of Prof. S. J. Przylecki, to whom I wish to express my sincere gratitude for his assistance during its prosecution.

# REFERENCES.

- Biltz and Schauder (1923). *J. prakt. Chem.* **106**, 108.  
 Flatow (1926). *Munch. med. Woch.* **73**, 12.  
 Garot (1926). *J. Physiol. Pathol. gen.* **24**, 779.  
 Piaux (1924). *Compt. Rend. Acad. Sci.* **178**, 782; **179**, 901.  
 Robinson (1924). *Biochem. J.* **18**, 255.  
 Warburg (1914). *Z. physiol. Chem.* **92**, 231.



## CLXIV. A NOTE ON VOLATILE SULPHIDE FROM MUSCLE.

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*(Received August 21st, 1928.)*

THE leg muscles of a well-nourished, recently killed guinea-pig were cut into small pieces by scissors and boiled in a distillation flask containing water, the delivery tube of which dipped below the surface of lead acetate solution. If the muscle was from a well-fed, sleek animal and was boiled immediately after the killing no volatile sulphide could be detected.

When some hours, preferably twenty-four, elapsed between killing the well-nourished animal and removal of the muscles, the presence of sulphide in the distillate was obvious. To remove the possibility of bacterial decomposition various procedures were adopted. In some the animals when killed were immediately eviscerated and the abdominal cavity was swabbed out with 1 % mercuric chloride solution. In others the amputated lower limbs were suspended, with skin intact or removed, in air containing toluene vapour. The results were the same in all cases. Apparently, therefore, autolysis makes a change in the muscle proteins whereby loosely bound sulphur is produced or liberated.

When the muscle was taken immediately after killing from a guinea-pig starved for 48 hours sulphide was readily detectable in the steam-distillate. These experiments were repeated many times and always with the same results. It is probable that the muscle of different animals shows considerable variation in this reaction. The flesh of sheep in poor condition emits sulphide readily on boiling but I have not succeeded in getting this with beef.

# CLXV. ON THE OXIDATION OF CHOLESTEROL BY MOLECULAR OXYGEN.

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*(Received August 18th, 1928.)*

## I.

WHILST the ability of the animal organism to synthesise cholesterol seems to be clearly proven, the question if, or to what extent, the cholesterol is broken down in oxidative or other ways in the body is still very obscure. This question is, indeed, not only of physiological interest, but in view of the diseases which are known to be accompanied by an abnormal accumulation of cholesterol in blood or tissues it is also of a definite pathological-clinical interest.

What is known about this question we owe to a great extent to Lifschütz [1927]. According to this author cholesterol is transformed by the action of permanganate or, better, benzoyl peroxide in acetic acid solution into a resinous product called "oxycholesterol." This substance can be recognised and estimated with the aid of a colour reaction with glacial acetic acid and sulphuric acid not given by the cholesterol itself. Since oxycholesterol has not been obtained crystalline and has no definite melting-point, the purity of the material is not yet assured. Lifschütz ascribes to it the formula  $C_{26}H_{47}O_2$ .

Oxycholesterol has been found by Lifschütz (and others) to be a normal constituent of the blood. It also seems to be present in certain other tissues. Lifschütz regards it as the first step in the break-down of cholesterol in the body. In spite of its indefinite chemical nature it merits, nevertheless, due attention, especially as a possible normal catabolite of cholesterol.

When protected from light pure cholesterol kept at room temperature seems to be very stable against the oxygen of the air. In the dry state it remains under such circumstances unchanged at least for weeks. Suspended in water it can be aerated for hours at 90–100° without perceptible changes (see below). Aeration of an alcoholic solution at 60–70° for a few hours at any rate (as we have found) does not affect it.

The mechanism by which the oxycholesterol is produced in the organism is not known. Lifschütz [1914] has found that oxycholesterol is formed when pure cholesterol in glacial acetic acid is heated for some days together with small amounts of dried, fat-free blood. He therefore is of the opinion that the primary oxidative attack on the cholesterol takes place in the blood. More recently Robertson [1925], in the course of experiments on the multiplication

of infusoria, found that cholesterol suspended in water in the presence of mere traces of substances from the acetone extract of brain tissue, when aerated at 100°, was oxidised to oxycholesterol. Perfectly pure cholesterol did not undergo oxidation under these conditions.

These observations of Robertson seemed to us to form a possible starting-point in the study of the mechanism by which the cholesterol is physiologically broken down. When we were already engaged in our work a paper was published by Moore and Willimott [1927] bearing on this subject. These authors found that when pure cholesterol in open dishes was heated to a temperature slightly above the melting-point it acquired after a few minutes a faintly yellow tint and afterwards gave a positive oxycholesterol reaction; they were not able to confirm Robertson's results that pure cholesterol suspended in water was stable when aerated at 90-100°. In such instances they also obtained invariably a positive oxycholesterol reaction. Moreover, pure suspensions without aeration directly evaporated at 100° gave similar positive tests. On the other hand the residues obtained after aeration for 48 hours at 37° with subsequent evaporation at the same temperature gave no coloration with the Lifschütz reagent. On account of these findings Moore and Willimott seem to be of the opinion that in the cases mentioned formation of resinous products with chromogenic properties similar to those of oxycholesterol is not due to an oxidation by oxygen but is an effect merely of heat. They recommend caution "in accepting the production of oxycholesterol as a specific effect of some particular mechanism in cases where heat is also involved."

That in the first of the above-mentioned observations the formation of oxycholesterol is due not merely to heat but also to the action of oxygen is, however, easily shown.

Two portions of pure cholesterol weighing each about 0.04 g. were heated at 150-155° for 20 minutes. Portion 1 was lying on an open watch-glass, portion 2 was placed in a small flask through which was led a very slow stream of pure nitrogen. After solidifying, portion 1 had a faintly yellow colour, was semi-transparent, and gave an intensively positive reaction for oxycholesterol. Portion 2 remained perfectly white and opaque and gave no trace of a positive Lifschütz reaction. When heated to about 155° in oxygen instead of in air the cholesterol acquired a yellow tint in a few minutes and showed a strongly positive oxycholesterol reaction. It was also found that if cholesterol suspensions, which, either "pure" or with an activating agent added, on aeration at 100° gave oxycholesterol, were treated with nitrogen at the same temperature, no oxycholesterol was formed.

The indispensable rôle of oxygen for the formation of oxycholesterol in these cases seems to be thus quite clear. The question next arises regarding the physical or chemical conditions necessary for the oxidation of cholesterol suspended in water by molecular oxygen. The following investigations are to be considered mainly as an inquiry into this question. Our experiments have been restricted to simple chemical systems.

## II.

At first our experience of the formation of oxysterol in "pure" suspensions was similar to that of Moore and Willimott. We had at our disposal a sample of cholesterol prepared in this institute from human gall stones. The preparation was perfectly white and gave no reaction for oxysterol. The melting-point was not determined. 0.5 and 1 % suspensions of this cholesterol were prepared by dissolving the substance in a little alcohol and pouring the solution with stirring into water heated to 60–70°. The suspensions were aerated at 90–100° with and without addition of acetone extract from brain tissue for 30 minutes up to 5 hours. No consistent results were obtained. In most cases the residues gave a strongly positive reaction for oxysterol. Occasionally, however, negative reactions were obtained, also in cases where brain extract had been added. In order to get reproducible results we tried to standardise as far as possible the experimental conditions, and to avoid impurities from different sources. Accordingly we submitted the cholesterol to purification by recrystallising it seven times from alcohol. Thereafter it was no longer possible to get suspensions of the same strength as before, since most of the cholesterol flocculated immediately. In order to obtain as concentrated suspensions as possible we followed the directions given by Svedberg [1924], who emphasises the importance of boiling off the solvent completely to obtain stable colloids. Accordingly, the alcoholic solution of the cholesterol was ejected in small portions into boiling water through the end of a glass tube of fine bore. Most of the alcohol thereby immediately volatilised. The rest was driven off by distilling the fluid to half the bulk, but we did not succeed in obtaining more concentrated suspensions than could be obtained by simply pouring the alcoholic solution with stirring into hot water. The highest concentrations attained were 0.07–0.08 %.

Even after the measures mentioned oxysterol was formed on aeration of "pure" cholesterol suspensions at 90–100°. The results, although less irregular than in the first series of experiments, were, however, quantitatively not reproducible. The cholesterol was then further purified in different ways: (1) by bromination and subsequent reduction with zinc dust, (2) by boiling for one hour with 0.5 *N* alcoholic potassium hydroxide, diluting with water, extracting with ether and taking up the residue after evaporation of the ether in light petroleum, (3) by boiling an alcoholic solution of cholesterol twice with blood charcoal (Merck's pure) for half an hour. After these purifications the cholesterol was once recrystallised from alcohol and then aerated at 90° for one hour. In all instances the Lifschütz reaction of the residue was positive. Considering the possibility that traces of oxysterol (or decomposition products of it) might favour its production by autocatalysis the residues obtained by evaporating the mother-liquors from the above-mentioned recrystallisations were tested for oxysterol. They all gave a positive reaction. The cholesterol purified with charcoal was then further recrystallised from

alcohol (4 times) until the residue from the mother-liquor no longer gave any reaction for oxycholesterol. The suspensions made with cholesterol thus prepared could be aerated for several hours (up to 7) without production of oxycholesterol in demonstrable amounts or, sometimes, with the formation of oxycholesterol in amounts giving only a faintly positive reaction. The method of purification at last adopted was the following. The crude cholesterol from gall stones was, after four recrystallisations from alcohol, boiled twice with blood charcoal in alcoholic solution for half an hour. The cholesterol was then further recrystallised from alcohol (3 or 4 times) until the residue from the mother-liquor no longer gave any positive test for oxycholesterol. The preparation thus obtained melted at  $148^{\circ}$  (corr.). Whether oxycholesterol actually acts autocatalytically was not further investigated. All that can be said is that the mode of purification above described proved to be reliable in repeated experiments for our purpose<sup>1</sup>. The highest concentrations of the suspensions prepared from this cholesterol were from 0.05 to 0.06 %.

### III.

In the course of this investigation we made an observation which seemed to us to be worth closer study. If merely a trace of an alkali soap was added to the cholesterol suspension we found regularly an abundance of oxycholesterol on aeration at  $90^{\circ}$ .

#### *Experimental.*

(1) The suspensions were prepared either according to Svedberg or simply by dissolving 0.05 g. cholesterol in 10 cc. alcohol and pouring the solution with stirring into 100 cc. water at  $60-70^{\circ}$ . In the earlier experiments we distilled off all the alcohol, but later found that this was not necessary. In some instances the long boiling necessary to drive off the alcohol seemed to make the suspension more prone to the formation of oxycholesterol, but with suspensions prepared in this way we obtained residues giving no reaction for oxycholesterol after aeration at  $90^{\circ}$  up to 7 hours.

(2) For the aeration 20 cc. of the suspensions were placed in a wide test-tube closed with a rubber stopper provided with three holes, respectively for inlet and outlet of air and for a thermometer. The glass tube for the inlet of air was in its lower submerged end enlarged to a bulb perforated with small holes. The outlet tube was connected with a reflux condenser in its turn connected with a suction pump. The air was in most experiments purified by filtering through cotton wool and by passing through three wash-bottles containing strong alkali, concentrated sulphuric acid and water respectively. The test-tube was immersed in a water-bath heated to the desired temperature. When the air was sucked through the suspensions in the boiling water-bath

<sup>1</sup> It was found that if the cholesterol thus purified was again boiled with charcoal the residue from the mother-liquor in the first recrystallisation from alcohol gave a positive reaction. Obviously small amounts of oxycholesterol are formed by the boiling with charcoal.

the temperature of the former sank, of course, below 100°, the more rapid the air stream, the lower the temperature. The temperature of the suspension therefore served as a relative measure of the rate of the air stream. By regulating the suction pump so that the temperature of the suspension was constantly at 90° we had a simple means of getting approximately the same rate of air stream in the different experiments at this temperature.

(3) The evaporation of the suspensions was performed in porcelain dishes placed in an air stream at room temperature. As the soaps interfere with the Lifschütz reaction the residues were extracted with light petroleum (B.P. 35–55°) and the latter was thereafter driven off. All vessels used were carefully cleansed with hot sulphuric acid-dichromate mixture.

(4) In some experiments estimation of the oxycholesterol was made roughly by direct inspection of the intensity of the colour reaction, in others by spectrophotometrical determination. A sample of oxycholesterol, prepared according to Lifschütz by oxidation of cholesterol by benzoyl peroxide, served as a standard. To 1 cc. of a chloroform solution of oxycholesterol was added 2 cc. of the reagent. Instead of adding, as Lifschütz did, the ferric chloride afterwards, we dissolved this substance in a concentration of 1/1000 directly in the reagent (9 vol. glacial acetic acid and 1 vol. conc. sulphuric acid). The spectrophotometrical reading was made after the mixture had been left for 15 minutes at room temperature. The spectral range used was that between 625 and 645 $\mu$ . The relation between the concentration of the oxycholesterol and the extinction coefficient was determined in a series of oxycholesterol solutions of varying concentrations. The spectrophotometer used was one of Hüfner's type.

The sodium stearate and sodium oleate used were pure preparations from Merck, the rest of the soaps tried were prepared by adding the calculated amount of alkali to the free fatty acids (Kahlbaum's). Table I shows the amount of oxycholesterol formed on addition of sodium stearate in varying concentrations. The suspensions were aerated for one hour at 90°. Repeated control experiments (without soap) at 90° and lower temperatures gave negative results. The amount of oxycholesterol formed is given as a percentage of the amount of cholesterol taken (the difference in the molecular weights referable to the oxygen uptake not being taken into consideration).

Table I.

Stearate concentration	Oxycholesterol formed
%	%
0.02	71
0.02	52
0.004	45
0.004	65
0.001	49
0.001	42
0.0002	5
0.0002	0
0.0002	0

Table II shows the production of oxycholesterol on aeration at different temperatures.

Table II.

A. Stearate concentration 0.004 %			B. Stearate concentration 0.02 %		
Aeration (hours)	Temperature	Oxycholesterol formed %	Aeration (hours)	Temperature	Lifschütz reaction
1	90°	65	1	90°	Strongly positive
1	85°	65	1	70°	Negative
1	80°	40	2	70°	Positive
1	75°	25	4	70°	Strongly positive
1	70°	10	1	60°	Negative
1	65°	0	3	60°	Faintly positive
1	65°	0	6	60°	Strongly positive
			3	55°	Negative
			6	55°	"
			12	55°	Positive
			12	45°	Negative

Tables III and IV show series of similar experiments with addition of sodium oleate. The controls were negative.

Table III.

Aeration for 1 hour at 90°.	
Oleate concentration %	Oxycholesterol formed %
0.02	57
0.02	57
0.02	98
0.004	25
0.004	14
0.004	25
0.001	0
0.001	13
0.001	10
0.0002	0
0.0002	0

Table IV.

Oleate concentration 0.02 %.		
Aeration (hours)	Temperature	Lifschütz reaction
1	90°	Strongly positive
1	80°	Positive
3	80°	Strongly positive
1	70°	Negative
3	70°	"
6	70°	"
9	70°	Positive

No close reproducibility as regards the quantitative data could be obtained. Qualitatively the results are unequivocal. On aeration at 90° the addition of 0.001–0.02 % sodium stearate or 0.004–0.02 % sodium oleate regularly brings about a rapid formation of oxycholesterol. The residues obtained in these cases were quite transparent and only very faintly yellow. Higher soap concentrations were not tried on account of excessive foaming. If the temperature is lowered the rate of oxycholesterol formation is also lowered. There

seems to be a steep fall of the rate of oxysterol formation in the stearate experiments at 60–55° and in the oleate experiments at 80–70°, which is probably not compatible with a normal temperature coefficient of the rate of the reaction.

In a few experiments with sodium palmitate, potassium palmitate and potassium stearate no oxysterol was at first formed on aeration at 90°. As other experiments had shown that the addition of alkali inhibited the effect of sodium stearate on oxysterol formation, we added small amounts of sulphuric acid to the soap solutions used (the amount of acid being so small that the fatty acids were not precipitated and the solutions were still faintly red to phenolphthalein). After the addition of acid the soap solutions brought about a liberal formation of oxysterol, while the addition of alkali in excess also, in the case of these soaps, inhibited the oxysterol production.

#### IV.

The observation of the marked effect of the soaps led us to investigate the effect of adding other hydrophile colloids. Thus a series of proteins (glutin, edestin, ovomucoid, peptone) was tried, further two carbohydrates, dextrin and gum arabic and finally unsaturated phosphatides from egg-yolk. Of these substances the ovomucoid and the phosphatides were prepared by ourselves, the others were commercial specimens. The concentrations used were the same as in the soap experiments. The aerations were carried out at 90° and also at lower temperatures. In some experiments the hydrogen ion concentration was varied by addition of small amounts of acid or alkali. In no instance was any formation of oxysterol promoted. The addition of a protein to a cholesterol suspension was, indeed, found to inhibit the effect of soaps.

The easy oxidation of the cholesterol at temperatures above its melting-point led us to ascertain whether, in suspensions containing in the colloid state substances in which cholesterol is soluble, the latter was possibly prone to oxidation by oxygen. Accordingly mixed suspensions of different triglycerides and of higher fatty acids and cholesterol were prepared. In no instance was oxysterol formed on aeration at 90°. (As the fatty acids and the fats interfere with the Lifschütz reaction, the residues were in these cases dissolved in 5–10 cc. alcoholic sodium hydroxide, the alcohol was evaporated to dryness on the water-bath with exclusion of air and the residues were extracted with light petroleum to separate the soaps.) No oxysterol was formed on aeration of a mixed cholesteryl oleate-cholesterol suspension.

Considering the well-known rôle of many heavy metal salts as catalysts in oxidation reactions we tried the effect of the addition of small amounts of ferric chloride, ferrous sulphate and cupric chloride. As the cholesterol suspensions are very easily precipitated by addition of such salts, they must be used in very low concentrations. In a strength of 1/100,000 they had no effect on the formation of oxysterol in pure suspensions. On the addition of ferric chloride in 0.005 % and 0.001 % concentrations to cholesterol suspensions



containing 0.02–0.04 % phosphatides we observed a formation of oxycholesterol at room temperature and without aeration. The amounts of oxycholesterol formed were relatively small (5–25 %) and irregular, so that we do not regard these results as very definite. Aeration and higher temperature did not increase the oxycholesterol formation in these cases.

## V.

The experimental results direct the attention to a certain physical factor which to all appearance is of the greatest importance for the attack of oxygen. It is very improbable that the soaps actually partake in the oxidation reaction. On the other hand the alkali soaps doubtless profoundly influence the colloidal state of the cholesterol. Simple inspection gives clear evidence of this fact. On adding (*e.g.*) 0.02 % sodium stearate to a 0.05 % cholesterol suspension, the mixture, when slowly heated, gradually becomes clearer. There is a distinct diminution of the turbidity at 40°, between 50–60° there is a further very marked clearing of the suspension, and at 60° it is nearly water-clear. Its turbidity equals then approximately that of a pure 0.05 % cholesterol suspension diluted with 7–8 parts of water. The clearness of the suspension contrasts strikingly with the milky appearance of a pure cholesterol suspension at the same temperature. When a cholesterol suspension containing 0.004 % sodium oleate is slowly heated, the turbidity remains practically unchanged up to between 70° and 80°, when it is rapidly reduced. (A 0.02 % sodium oleate solution itself is still rather milky at 90°.)

It should be noted that at the same temperatures at which the clearing of the suspensions takes place, there is—especially in the oleate experiments—a marked increase in the rate of the oxycholesterol formation, an increase which can hardly be an ordinary temperature effect on the rate of the reaction. It may also be mentioned that none of the other colloids tried brought about any visible clarification of the cholesterol suspensions.

There can be no doubt that this marked clearing of the suspensions is referable in the main to an increased degree of dispersion of the cholesterol. This is in itself no remarkable occurrence, the powerful peptising action of the alkali soaps being a well-known phenomenon. The point lies in the observation that parallel with the increased degree of dispersion brought about by the soaps the oxidisability of cholesterol by oxygen is essentially increased<sup>1</sup>. This observation suggests that, provided that the cholesterol exists in a sufficiently high degree of dispersion in water, the substance may require no particular catalyst for a relatively rapid oxidation by oxygen even at body temperature. As the cholesterol in the blood normally exists in a high degree of dispersion, the results obtained support to a limited degree the opinion that cholesterol

<sup>1</sup> In heterogeneous systems the rate of the reaction is a function of the surface area between the phases containing the reacting substances. The degree of dispersion, therefore, strongly influences the rate of the reaction. It can, however, not be excluded that the soaps act catalytically also in some other way than by changing the degree of dispersion of the cholesterol.

may undergo oxidative changes in the blood. The observed inhibition of the soap effect by alkali and the resistance of cholesterol in alcoholic solution against oxygen suggest, *inter alia*, that other factors than the degree of dispersion are of decisive importance.

With regard to the question of the catalysing substances present in impure cholesterol, there are certain evidences of their acting in the same manner as the soaps. The fact that with impure cholesterol it was possible to prepare more concentrated suspensions than with the quite pure substance points to the presence of peptising agents in the impure preparations. The non-formation of oxycholesterol on aeration at 37° for 48 hours (in the experiment of Moore and Willimott) as compared with the liberal amounts of oxycholesterol formed on direct evaporation of the suspensions on the boiling water-bath indicates an abnormal temperature coefficient of the rate of the reaction in the same way as in the soap experiments. Furthermore, in our first series of experiments (p. 1315) with impure cholesterol we often noted a very marked clearing of the suspensions in cases where oxycholesterol was formed. As soaps are claimed to be normal constituents of the bile there is the possibility that the active impurities (in our preparations) were actually soaps. The active principle of the brain extract was not investigated.

As to the failure to get quantitatively reproducible results we think that it is most likely due to the difficulty to reproduce exactly the same colloidal state (or series of states) in the different experiments.

The observations made call for caution against the action of oxygen in the determination of oxycholesterol in blood or tissues. Finally the relative character of the qualitative results obtained with the Lifschütz reaction may be emphasised. It is clear that if greater quantities of the suspensions had been used in the aeration experiments than those employed by us, positive Lifschütz reactions might have been encountered in cases where we obtained negative ones, and conversely if smaller quantities of suspensions had been taken negative reactions might have been found where we got positive ones.

#### SUMMARY.

The formation of oxycholesterol on heating cholesterol in the air at temperatures slightly above the melting-point or on aeration of cholesterol suspensions is due to the action of the atmospheric oxygen. The observation by Robertson that suspensions of pure cholesterol are, even at boiling temperature, very stable against the oxygen of the air is confirmed. Impurities very stubbornly adhering to the cholesterol catalyse the formation of oxycholesterol on aeration. Alkali soaps have a marked catalysing effect on the oxidation of the cholesterol by molecular oxygen. A series of other hydrophile colloids tried had no catalytic action. The same was the case with a series of higher fatty acids, triglycerides and heavy metal salts. The action of the soaps in all probability is mainly due to their peptising action on the cholesterol. The catalysing impurities probably act in the same way. The observations

made call attention to the great importance of the colloidal state of the cholesterol—especially its degree of dispersion—for the oxidisability of the substance by molecular oxygen.

## REFERENCES.

- Lifschütz (1914). *Z. physiol. Chem.* **93**, 209.  
— (1927). *Arch. Pharm.* **265**, 450.  
Moore and Willimott (1927). *Biochem. J.* **21**, 585.  
Robertson (1925). *Austral. J. Exp. Biol. Med. Sci.* **2**, 83.  
Svedberg (1924). *Colloid chemistry*. (New York.)

## CLXVI. A NOTE ON QUANTITATIVE METHODS OF MEASURING THE NUTRITIVE VALUE OF PROTEINS.

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IN an article appearing recently in this *Journal*, Kon [1928] has applied to the study of the nutritive value of the protein tuberin, the globulin of the potato tuber, the method elaborated by the author [1924], involving a study of the nitrogen metabolism of young growing rats under controlled conditions of feeding. In comparison with this method, analogous results obtained by a quantitative growth method devised by Osborne, Mendel and Ferry [1919] were also presented. According to the first method, the nutritive value of the protein is taken as the total nitrogen retention of the experimental animal in percentage of the total absorbed nitrogen. This percentage is called the "biological value" of the protein, a term introduced by Thomas [1909]. According to the second method, the nutritive value of the protein is measured by the gain in body-weight of the experimental animal per gram of protein consumed.

In discussing the significance of the results secured by these two methods, Kon makes certain statements to which exception may be taken. Speaking of the nitrogen balance method, he says (pp. 266-7): "A specific error seems to be inherent in the method . . . namely, the tendency to give higher biological values in the periods immediately following the standardising nitrogen-free or low-nitrogen periods. This is especially marked in the present work, but may also be found in the papers of Mitchell and his co-workers, especially Mitchell and Beadles [1927], and of Klein, Harrow, Pine and Funk [1926]."

If this were true it would constitute a serious criticism of the method. Among the first applications of the method in the author's laboratory, the possibility of such an error was anticipated and specifically studied by Mitchell and Carman [1924], with results quite contrary to the conclusion of Kon. It should be realised in a consideration of this question, first, that discrepancies will generally exist between duplicate determinations of the biological value of a specific source of protein obtained with two groups of rats or even with the same group of rats, and, second, that if one of the duplicate determinations is made in the period following a period of low-nitrogen feeding, the chances are even that it will be larger than the other determinations not so made,

even though the period of nitrogen underfeeding has exerted no effect on the utilisation of the dietary protein. In other words, the question is a statistical one, and cannot be settled by citing isolated instances in which experimental data *appear* to indicate an increased biological value following nitrogen underfeeding. The results of Kon obtained with tuberin are of this description, but neither they, nor the other results cited, are sufficient to prove the point at issue, since they have been selected from a number of other results of an opposite significance. Thus, in the work of Klein *et al.* [1926], wheat bran gave an average biological value of 60 following a period of nitrogen-free feeding, and one of 54 in another type of period, but corn meal gave very poor and variable results (averaging only 18) in a period following a standardising period, and an average of 56 in another period not so located. The same situation exists in the work of Mitchell and Beadles [1927] to which reference is also made.

It seems clear that the best solution of the question can be obtained only from a survey of all published data that might bear upon it. Besides the Illinois data, there are only those data from Cambridge and Warsaw already cited. The following table summarises all of the data obtained from the author's laboratory at the University of Illinois.

*The effect of a period of low-nitrogen feeding on the biological value of protein obtained in an immediately following period.*

Source of protein	Average biological values obtained		Excess of (1) over (2)	Reference
	Following low-N feeding (1)	Not following low-N feeding (2)		
Whole egg	92	94	-2	Mitchell and Carman [1924]
Whole wheat	63	68	-5	"
Cocoa + milk	73	65	+8	Mitchell, Beadles and Keith [1926]
Whole egg	90	99	-9	Mitchell and Carman [1926]
White flour	56	48	+8	" " "
Egg albumin	86	81	+5	" " "
Egg albumin	82	81	+1	" " "
White flour	48	54	-6	" " "
Veal	64	59	+5	" " "
White flour	49	49	$\pm 0$	" " "
Beef + white flour	70	76	-6	" " "
White flour	55	55	$\pm 0$	" " "
Beef liver	78	75	+3	Mitchell and Beadles [1927]
Beef heart	84	68	+16	" " "
Beef heart	76	69	+7	" " "
Beef liver	73	75	-2	" " "
Pork tenderloin	79	74	+5	Mitchell, Beadles and Kruger [1927]

Among 17 possible comparisons, 9 favoured the view that low-nitrogen feeding increases the biological value in an immediately subsequent period; 6 contradicted this view, and 2 gave a non-committal answer. The average difference in biological value for the 17 cases was +1.6, in favour of the value obtained after low-nitrogen feeding. As the method is applied in the author's laboratory, there seems to be no reason to believe that a period of low-nitrogen

feeding exerts any but an inappreciable effect upon the utilisation of protein in a subsequent period.

Kon is also in error in his attempt to explain differences in the nutritive ratings of proteins as obtained by the author's method and by the method of Osborne, Mendel and Ferry. On this point he says: "Obviously the two methods are not strictly comparable, as the one measures the efficiency of a given protein to make good, over short periods of time, the wear and tear of body tissues, while the other measures the ability of a protein to build new tissues."

This constitutes a misinterpretation of the significance of results secured by the author's method, a misinterpretation that can be readily refuted by any of the calculations made by Kon himself. Take the data of Rat 1 on the tuberin ration, for instance. This rat consumed daily 104 mg. of tuberin-nitrogen and excreted daily in the faeces 23 mg. of nitrogen, of which 22 mg. were estimated to be of body origin (metabolic nitrogen). Hence, only 1 mg. of dietary nitrogen was actually indigestible, and the absorbed nitrogen, therefore, amounted to 103 mg. daily. The daily urinary nitrogen amounted to 45 mg., of which 20 mg. was estimated to be of body origin (endogenous) and only 25 mg. represented unutilised dietary nitrogen. Hence, of the 103 mg. of tuberin nitrogen absorbed 25 mg. was wasted and 78 mg., or 76 % (the biological value), was retained in the body. According to Kon, this 78 mg. was used only to make good the wear and tear of body tissues. But this rat was growing and storing daily 36 mg. of nitrogen ( $104 - 23 - 45 = 36$ ), which must be an integral fraction of the total retention of 78 mg., since no other source of nitrogen was available. The latter value includes all functions, maintenance, growth, and the replenishment of the digestive glands—for which the dietary nitrogen is being used.

Thus, the biological value of a protein measures that fraction of the absorbed protein nitrogen that is being used in all of the anabolic reactions of the body. On the other hand, the gain per gram of protein consumed obtained under the conditions prescribed by Osborne, Mendel and Ferry credits to the dietary protein only that fraction of the protein intake that is being used for growth. Hence, this measure of the nutritive value of a protein is affected, not only by the amino-acid make-up of the protein itself and the unknown physiological factors that determine its utilisation in anabolism, but also by the ratio of protein consumed to the size of rat used. The larger this ratio becomes, the smaller will be the fraction of the nitrogen intake needed to make good the endogenous losses of nitrogen, and the greater the fraction that is available for growth, and, hence, for the same source of protein, the greater the gain per gram of protein consumed. For this reason, this measure of protein value is subject to variation caused by variable food intakes. If rats of equal size are employed and the food intakes are comparable, the measures of protein efficiency obtained for different proteins will be comparable. But it is a hazardous undertaking to compare gains per gram of protein consumed

obtained in different experiments without reference to the size of animals employed, or to the amounts of food consumed, as Kon and others before him have done.

Hoagland and Snider [1926], in one of their first applications of the method of Osborne, Mendel and Ferry to the study of the nutritive value of proteins, noted that, throughout a number of ration comparisons, the gain in weight per gram of protein consumed was in the large majority of cases greater for the male than for the female rats. This sex difference was correctly interpreted as the result of the greater growth capacity of male rats and the resulting greater consumption of food in proportion to weight. Hence, with the males a larger proportion of the protein consumed was available for growth and was so used. Although in their later work Hoagland and Snider used only male rats, the complete significance of these early experiments has not been appreciated, *i.e.* that in comparisons of different sources of protein a great disparity in the food consumption of experimental rats, whether because of differences in sex or of the growth-promoting value of the rations, will always obscure the interpretation of the observed differences in the ratio of gain to protein consumed.

There is another reason why the results obtained by the author's method for a series of proteins will differ from those obtained by the numerical method of Osborne, Mendel and Ferry. In the former method differences in the digestibility of proteins are not involved in the biological values obtained, while in the results of the latter method they are. For two proteins of equal biological value, the gain in weight per gram consumed will be the greater for the more digestible protein. This is one reason why animal proteins are graded so much higher than cereal proteins by this method than they are by their respective biological values.

Either method is capable of yielding accurate comparisons of the nutritive values of different sources of protein, but only when the necessary experimental conditions are imposed, as dictated by a critical study of all factors that may affect the final results. However, the methods are not equivalent and the results obtained need not parallel each other.

#### REFERENCES.

- Hoagland and Snider (1926). *J. Agric. Res.* **32**, 1025.  
Klein, Harrow, Pine and Funk (1926). *Amer. J. Physiol.* **76**, 237.  
Kon (1928). *Biochem. J.* **22**, 261.  
Mitchell (1924). *J. Biol. Chem.* **58**, 873.  
Mitchell and Beadles (1927). *J. Biol. Chem.* **71**, 429.  
Mitchell, Beadles and Keith (1926). *J. Biol. Chem.* **71**, 15.  
Mitchell, Beadles and Kruger (1927). *J. Biol. Chem.* **73**, 767.  
Mitchell and Carman (1924). *J. Biol. Chem.* **60**, 613.  
——— (1926). *J. Biol. Chem.* **68**, 183.  
Osborne, Mendel and Ferry (1919). *J. Biol. Chem.* **37**, 223.  
Thomas (1909). *Arch. Anat. Physiol.* 219.

## CLXVII. OXIDISING ENZYMES.

### X. THE RELATIONSHIP OF OXYGENASE TO TYROSINASE.

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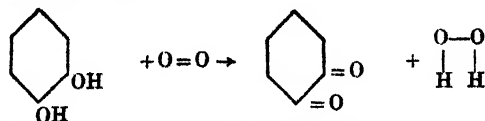
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*(Received September 3rd, 1928.)*

IN a recent communication [Pugh and Raper, 1927], the following statement has been made: "tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative.... There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow." The present communication records some observations made in connection with the above statement.

It is known that a preparation from the potato and many other Higher Plants giving the "direct oxidase" reaction made according to Onslow [1920], that is, by thorough extraction of the tissues with cold alcohol without undue oxidation, and subsequent extraction of the enzymes with water from the residue, will rapidly convert catechol and other *o*-dihydroxy-substances into *o*-quinones [Szent-Györgyi, 1925; Pugh and Raper,<sup>1</sup> 1927] and the monophenols, phenol, *p*-cresol, *m*-cresol and tyrosol, and *p*-hydroxybenzoic acid [Onslow and Robinson, 1925; Uys-Smith, 1926; Pugh and Raper,<sup>1</sup> 1927] and tyrosine [Onslow, 1923; Raper,<sup>2</sup> 1926] into the corresponding *o*-dihydroxy-compounds, with the subsequent formation of *o*-quinones. Such a preparation also contains peroxidase

It is known [Onslow, 1920] that if an aqueous solution of catechol is exposed to air, it autoxidises with the formation of a peroxide, since it reacts with peroxidase and guaiacum. As far as qualitative tests are valid, the peroxide appears to be hydrogen peroxide on account of the reaction given with titanium sulphate [Onslow and Robinson, 1926; Platt and Wormall, 1927]. It is possible that *o*-quinone and hydrogen peroxide are formed according to the equation below:



<sup>1</sup> The *o*-quinone was identified by Raper using tyrosinase from the meal-worm, and catechol, phenol, *p*-cresol and *m*-cresol as substrates.

<sup>2</sup> 3 : 4-Dihydroxyphenylalanine was also identified by Raper in this reaction, again using tyrosinase from the meal-worm.



The *o*-quinone here, however, cannot be detected by guaiacum. It has been suggested [Szent-Györgyi, 1925] that it may condense under certain conditions with unchanged catechol.

Such a reaction now appears to represent that carried out by the enzyme, oxygenase, of the Higher Plants, the *o*-quinone having recently been isolated by Pugh and Raper (as stated above), who have, moreover, shown that the same product is formed from catechol by the action of peroxidase (horse-radish) and hydrogen peroxide.

Since such oxygenase plants as have been examined also contain a catechol derivative [Onslow, 1921], it is reasonable to assume that the above is the reaction which takes place, with simultaneous browning, on injury to the cell.

Now, the preparation as described by Onslow contains oxygenase and peroxidase, both of which can carry out the reaction denoted above. It is obvious, moreover, that such a preparation may always contain traces of *o*-quinone, though not sufficient to blue guaiacum, and possibly of catechol. The oxidation of catechol in the presence of such a preparation is therefore due to both oxygenase and to peroxidase and hydrogen peroxide; in addition, the oxygenase itself might be regarded essentially as the *o*-quinone, perhaps stabilised by association with a colloidal surface. The question also naturally arises as to whether the oxidation of the monophenols is brought about by the same enzyme as that which catalyses the oxidation of *o*-dihydroxy-compounds or by another catalyst; or whether it is a secondary oxidation in which *o*-quinone, formed from the diphenols originally present, takes part. Bearing in mind, therefore, that the *o*-quinone may be an intrinsic part of the oxidising system, we have tried experimentally to obtain some evidence on this point.

On this assumption, a plant tissue which has been allowed to oxidise should yield a more active enzyme preparation than one which has not received such treatment, because, in the former, more *o*-quinone will presumably be present. We therefore took two small portions (about 20 g. each) from the same potato. One portion was coarsely pounded in a mortar and allowed to oxidise for about 10 minutes; it was then thoroughly ground and extracted five times with alcohol and air-dried. The other portion was very rapidly sliced under alcohol with maximum care to avoid contact with air, and immediately ground and extracted with alcohol five times and air-dried. Equal portions of both preparations were then treated with equal volumes of freshly-prepared 0.1 % aqueous catechol, 0.1 % *p*-cresol in phosphate buffer ( $p_H$  7.3), and a saturated solution of tyrosine in the same buffer. The oxidised preparation acted more rapidly on all three substances, the differences in rate being small but definite for catechol, and greater for *p*-cresol and tyrosine. We have so far been unable to maintain this difference on reprecipitation of the two enzyme preparations from aqueous solution by alcohol.

In addition, we have tried the effect of treatment of the enzyme preparation with charcoal, with the object of removing the traces of *o*-quinone present. About 75 g. of potatoes were pounded, extracted four times with alcohol and

air-dried. The residue was then extracted with 50 cc. of water, and filtered, about 40 cc. of extract being obtained. Preliminary tests with freshly prepared aqueous catechol, and with *p*-cresol and tyrosine, both in buffer solution, were made; the solution was then shaken up five times with Merck's charcoal (0.1 g. per 5 cc.), portions being tested between each adsorption. The results obtained are set forth in the following table.

Solutions tested with 0.5 cc. enzyme extract		Extract after 1st adsorption					
		Original extract	with charcoal	After 2nd adsorption	After 3rd adsorption	After 4th adsorption	After 5th adsorption
0.1 % catechol	1 cc.	Instan- taneous	Instan- taneous	Instan- taneous	Instan- taneous	1 min.	3 min.
0.1 % <i>p</i> -cresol	1 cc. }	3 min.	42 min.	55 min.	85 min.	Negative	Negative
Water	1 cc. }					after	after
						2 days	2 days
0.1 % <i>p</i> -cresol	1 cc. }	—	4 min.	5 min.	8 min.	23 min.	> 1 hour
0.0005 % catechol	1 cc. }						colour
							fainter
Sat. solution tyrosine	1 cc. }	50 min.	Negative	Negative	Negative	Negative	Negative
Water	1 cc. }		after				
			2 days				
Sat. solution tyrosine	1 cc. }	35 min.	6 min.	10 min.	15 min.	> 1 hour	Negative
0.0005 % catechol	1 cc. }						

The times recorded refer in each case to the first appearance of a definite colour.

It will be seen from the table that the onset of oxidation of catechol was not obviously retarded until after the fourth adsorption, although the intensity of the reaction, as indicated by the colour, gradually decreased. The oxidation of *p*-cresol, on the other hand, was diminished very considerably after the first adsorption and eventually ceased, whereas it was restored to practically the original value by the addition of about 0.005 mg. of catechol. The extract was unable to bring about the oxidation of tyrosine after the first adsorption; this capacity was restored to a small extent by the addition of traces of catechol; unlike, however, the case of *p*-cresol, oxidation does not proceed normally, a very small amount of the tyrosine present being oxidised.

From the observations recorded above, we conclude that the activity of the colloidal constituent is at first almost unimpaired by charcoal, as shown by the continued rapid reaction with catechol. On the other hand, the oxidation of *p*-cresol is greatly retarded by the first adsorption with charcoal, and gradually ceases with successive treatments; it is, moreover, restored to almost its initial strength by the addition of a trace of catechol. It may be assumed, therefore, that it is a secondary oxidation depending on the presence of *o*-quinone, which has been largely removed by the first, and probably completely so, by the fifth treatment. It seems as if the addition of a trace of catechol may result in the production of *o*-quinone, which initiates the autocatalysis following on the induced oxidation with production of homocatechol from *p*-cresol.

In the case of tyrosine, induced oxidation seems to take place to a certain

extent, after adsorption, on the addition of traces of catechol, as the solutions turn pale pink and darken slightly; but, as the reaction never increases in intensity, autocatalysis apparently does not proceed far under these conditions. It is known from the work of Raper [1927], that further oxidations take place in the formation of melanin from tyrosine, and the reaction is obviously more complicated than in the case of *p*-cresol.

Peroxidase has always been detected in our enzyme extracts after the last adsorption. The presence of peroxidase has a certain significance in the experiment, for, as stated earlier in the paper, a solution of catechol slowly autoxidises in air, producing, in all probability, hydrogen peroxide. In the presence of the latter and peroxidase, *o*-quinone is formed from catechol. The difference in effect between a freshly prepared catechol solution and one which has stood for 24 hours or more could be demonstrated, not only with the original enzyme extract, but also after each successive adsorption, the colour appearing slightly earlier with the autoxidised catechol.

It appears to us to be certain that there is an enzyme, present in plants giving the "direct oxidase reaction," which catalyses the autoxidation of *o*-dihydroxy-compounds with the probable production of hydrogen peroxide and *o*-quinone; the plants which give the "peroxidase reaction" only are incapable of originating such a catalysis. We see no reason for discarding the term oxygenase for this enzyme, or for substituting the term tyrosinase for an enzyme which primarily catalyses the oxidation of substances with the *o*-dihydroxy-grouping. From the evidence given in this paper, it seems highly probable that the *o*-quinones produced from such substances will bring about, as a secondary oxidation, the formation of dihydroxy-compounds from monophenols, such as *p*-cresol. To a slight extent the *o*-quinone can initiate the formation of melanin from tyrosine, but the fact that the oxidation is not greater in extent may indicate that some additional factor is concerned in the normal reaction. It seems, nevertheless, that the term, tyrosinase, may, when the conditions are definitely realised, ultimately come to be interpreted as oxygenase plus an *o*-dihydroxy-compound or its derived quinone. If, in addition, it should be proved that the oxygenase itself is really an *o*-quinone in conjunction with a colloidal surface, then the original conception of Bach and Chodat [Chodat, 1910] of the oxygenase as "an enzyme-like peroxide" would be vindicated, though re-expressed in more modern terminology. Throughout the present paper, our remarks only apply to enzymes of the Higher Plants. Further, we have been unable to demonstrate a tyrosinase reaction in any plant which did not also contain the catechol-oxygenase system. In regard to the polyphenolase and laccase oxidising systems of the Higher Plants, our view is that these terms are synonymous with the oxygenase-catechol system. It is true that certain laccases of the Higher Plants [Bertrand, 1896] have been stated to have no action on tyrosine. We also have found a few cases of oxygenase-catechol plants not acting on tyrosine; but, as the conditions of the reaction are not completely understood, they cannot yet be

established as definite exceptions. We have retained the name, oxygenase, on account of the classical terminology of Bach and Chodat. Until it should be known definitely that oxygen is not activated in the reaction, we see no reason for substituting another term.

Concerning the classification of aerobic oxidases proposed by Pugh and Raper [1927], we would like to point out, in connection with system (1), namely peroxidase plus an autoxidisable substance [Gallagher, 1923], that a criticism of the interpretation of this author's observations has been published by one of us [Robinson, 1924]. The work of Euler and Bolin [1908], which concerns the proposed system (3), has been criticised by Bunzel [1915]. System (4), tyrosinase, we prefer to interpret on the lines indicated above, where we have expressed our views as to the connection of this enzyme with the catechol-oxygenase, the polyphenolase and the laccase systems.

#### REFERENCES.

- Bertrand (1896). *Compt. Rend. Acad. Sci.* **122**, 1215.  
 Bunzel (1915). *J. Biol. Chem.* **20**, 697.  
 Chodat (1910). *Handb. biochem. Arbeitsmeth.* Abderhalden (Berlin), **3** (i), 42.  
 Euler und Bolin (1908). *Z. physiol. Chem.* **57**, 80.  
 Gallagher (1923). *Biochem. J.* **17**, 515.  
 Onslow, M. W. (1920). *Biochem. J.* **14**, 535.  
 — (1921). *Biochem. J.* **15**, 107.  
 — (1923). *Biochem. J.* **17**, 216.  
 Onslow and Robinson (1925). *Biochem. J.* **19**, 420.  
 — (1926). *Biochem. J.* **20**, 1138.  
 Platt and Wormall (1927). *Biochem. J.* **21**, 26.  
 Pugh and Raper (1927). *Biochem. J.* **21**, 1370.  
 Raper (1926). *Biochem. J.* **20**, 735.  
 — (1927). *Biochem. J.* **21**, 89.  
 Robinson (1924). *Biochem. J.* **18**, 543.  
 Szent-Györgyi (1925). *Biochem. Z.* **162**, 399.  
 Uys-Smith (1926). *Biochem. Z.* **168**, 448.

## CLXVIII. GLUCOSE IN NORMAL URINE.

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*(Received July 13th, 1928.)*

### INTRODUCTION.

IN spite of considerable investigation, the question whether small amounts of glucose may occur in normal urine has not yet been satisfactorily answered. The present state of indecision is partly due to the absence of a specific test for this sugar and partly to the fact that it normally occurs in very small quantities.

Apart from reduction tests the detection of glucose has generally been based on (1) fermentation with yeast, and (2) the phenylhydrazine reaction. That the reaction is not specific for glucose is not the only drawback as far as yeast fermentation is concerned, for yeast [von Lipmann, 1904] fails, sometimes, to ferment sugar when the concentration of the latter is as low as 0.1 g. per 100 cc. During the present investigation this observation was confirmed. It was possible to obtain glucosazone from urines to which 0.1 g. glucose per 100 cc. had been added even after 3 days' incubation at 37° with a pure yeast culture [see also Patterson, 1926].

Objections have often been raised against the use of yeast for the detection of glucose in normal urine. The laborious technique, which the use of pure cultures of yeast necessitates, has unfortunately led to the employment of ordinary yeast instead. This practice has no doubt weakened the evidence for the existence of glucose, though in the majority of urines thus treated fermentation did occur [Benedict and his co-workers, 1918; Sumner, 1921; Neuwirth, 1922]. But, as has frequently been suggested, such fermentation might be due to the bacteria introduced with the yeast. To attempt to inhibit bacterial growth by making the urine slightly acid or by adding a little volatile antiseptic like toluene is of doubtful value especially if, as is customary, fermentation with ordinary yeast is allowed to go on for 17 to 24 hours or more. In spite of starting with a  $p_H$  varying between 5.5 and 6.8, Höst [1923] found that some of the urines undergoing fermentation with ordinary yeast became strongly ammoniacal. Much reliance cannot, therefore, be placed on positive evidence obtained in experiments with yeast if bacteria were present.

The osazone reaction properly carried out is free from such serious drawbacks, and it may, therefore, be expected to furnish more reliable information regarding the question of glucose in normal urine. As has been pointed out

by Höst [1923], the only sugar capable of giving an osazone identical with that of glucose is laevulose, but the optical behaviour of normal urine seems to show that laevulose is most probably not one of the urinary sugars. Moreover, observations reported by Folin and Berglund [1922] suggest that, unlike galactose and lactose, laevulose ingested even in large quantities is retained in the body.

The belief that glucose is a constituent of normal urine arose from observations made by Baisch [1894] and confirmed by Lemaire [1895] and by Pavy and Siau [1901]. These investigators isolated from normal urines an osazone apparently identical with glucosazone. Recently, however, Geelmuyden [1915] and Höst [1923], who applied the same test to small quantities of urines, were unable to confirm the results arrived at by these earlier investigators, though many normal urines were examined.

Dissatisfied with the methods so far employed, Folin and Berglund [1922] attempted to throw fresh light on the problem by studying the relation between sugar elimination and the concentration of sugar in the blood. The blood-sugar and the urine-sugar were determined before and after hydrolysis under various conditions and it was found that giving as much as 200 g. of pure glucose to normal persons was not followed by any glucosuria, whereas definite "glycuresis" was obtained after every ordinary carbohydrate meal. These observers ascribe the increase in the reducing substances of the urine after an ordinary meal to the excretion of foreign unassimilable carbohydrates and carbohydrate decomposition products produced during the preparation of the food. According to them glucose is probably not one of the urinary sugars. Similar conclusions were arrived at by Höst [1923] and by Greenwald and his co-workers [1925].

The present paper records certain observations which were made while applying the osazone test to a number of urines mainly from healthy Egyptian students and which provide confirmation of the older view that glucose may be a constituent of normal urine.

## EXPERIMENTAL.

### 1. *The preparation of the osazones of the urinary sugars.*

Normal urines contain substances that interfere with the proper crystallisation and consequently with the identification of the osazones of the urinary sugars. Even when the concentration of the sugars is so large that recognisable crystalline osazones come down when the urine is treated with phenylhydrazine, the crystals are, as a rule, ill-formed, dirty and often buried in an amorphous deposit of brown particles and oily drops. The substances which thus interfere with the formation and crystallisation of osazones can be removed quite readily by treating the urine with adsorbent charcoal before applying the phenylhydrazine test.

In all the experiments recorded in this paper, the urines were treated in this manner and evidence was obtained that charcoal removes these interfering substances without appreciably lowering the concentration of the urinary sugars. The charcoal employed was usually unpurified Merck's decolorising charcoal. The following method was found suitable for the preparation of osazones from normal urines.

25 cc. urine were well mixed with 2 g. dry charcoal and filtered after standing for about 5 minutes. About 15–17 cc. clear colourless filtrate was usually obtained, and to this was added 1 g. of a mixture of pure phenylhydrazine hydrochloride and sodium acetate (prepared by mixing well in a mortar one part of pure phenylhydrazine hydrochloride and two parts of sodium acetate just before use). The filtrate was now placed in a boiling water-bath for about 5 minutes or just until the reagents were dissolved and then filtered into another clean tube. The final filtrate was put back in the bath for 2 hours and then allowed to cool gradually in the bath and the deposit examined for crystals 12–15 hours later.

As described above the reaction is not only sensitive but seldom fails to yield clean and well-crystallised osazones. Occasionally urines of low specific gravity fail to give crystals at first, but most of these do so on longer standing at a temperature of 3–5°.

Sometimes the crystals are contaminated with variable amounts of orange-brown oily drops which, whilst in no way interfering with the delicacy of the test, may tend to obscure their appearance under the microscope. This impurity can be effectively removed by washing the deposit two to three times with a little ether after the removal of the supernatant liquid.

#### *The sensitivity of the osazone test.*

To test the delicacy of this reaction for the detection of glucose, it was applied, in the form described above, to solutions of glucose in water and in urine.

*Glucose dissolved in water.* From aqueous solutions of glucose it was a simple matter to obtain typical glucosazone crystals when the concentration of glucose was 2.5 mg. per 100 cc. This confirms the observations of H $\ddot{o}$ st [1923] who employed a modified Neumann's method. The reaction is, however, more sensitive than he imagined, for by cooling the tubes to 3–5° for a few hours, aqueous solutions containing as little as 1 mg. glucose per 100 cc. were found to give crystals of glucosazone.

*Glucose added to urine.* The presence in urine of substances which interfere with the osazone test, as well as the fact that all urines examined yield osazones, make it difficult to determine the delicacy of the test for the detection of added glucose until the urinary sugars have been removed. This was accomplished by bacterial fermentation in the following way.

The urine is made just alkaline with ammonia, inoculated with a few drops from another urine undergoing active alkaline fermentation and incubated at

37° for 10–12 hours. It is then acidified slightly with acetic acid, a sufficient quantity of washed kieselguhr added to assist the flocculation of the bacteria and brought up to the boil. After cooling, it is filtered and the clear filtrate is used for the test. Such urine will not give an osazone.

Tests on such urines with added glucose demonstrated that as little as 1 mg. per 100 cc. can be detected by the osazone test.

*The nature of the osazones derived from normal urine.*

The crystalline forms of the osazones obtained from normal urines are very varied (Plate V, Figs. 1–7). Nevertheless, it can be shown that they usually consist of mixtures of at least two osazones which can, in most cases, be separated by simple means. The crystals may be composed entirely either of fine needles or of broad flat needles, but more frequently both kinds are combined to form one crystal mixture (Figs. 2, 3 and 4). In some, however, their mixed nature is not quite so clear. Union between the two osazones apparently takes place before crystallisation and the resulting crystal possesses new characters, but these are always intermediate between those of the components (Fig. 5). A more complex type of crystal is frequently met (Fig. 6) but this is simply an aggregate of crystals belonging to the two classes of the crystal mixture just described.

The appearance of crystals in Figs. 2, 3, 4 and 6 indicates that they are a mixture of the two osazones represented by Figs. 1 and 7. This can be proved by fractional crystallisation, since these two osazones differ markedly in solubility.

The osazone deposit from 200 cc. of a 24 hours' fresh urine was washed with distilled water and then with ether, 10 cc. of 20 % alcohol were then added and after stirring well, the mixture was centrifuged in a corked tube. The clear orange-brown supernatant liquid was then drained off and kept aside and the residue examined microscopically. This residue was dissolved in a little hot absolute alcohol and distilled water carefully added to the solution until a slight permanent turbidity occurred. The solution was then boiled to drive off some of the alcohol and on cooling fine long needles were obtained, many of which remained single, others formed broom-like aggregates. Microscopically these crystals were identical with glucosazone.

The clear supernatant liquid was treated with a little ether, and the top ether-alcohol layer pipetted off. This on recrystallisation gave a mixture of crystalline forms in which were recognised (a) a small glucosazone sheaf, (b) a larger irregular mixed osazone sheaf, and (c) two separate broad flat needles.

Crystals containing comparatively little glucosazone give after such treatment a fair yield of the osazone which crystallises out in broad flat needles,

The fractional crystallisation of many similar crystals obtained from urine gave only the following two simple osazones.



*Osazone I.* This is characterised by crystallising in broad flat orange-yellow needles of which the ends usually taper gradually, or in some cases form blunt spear-shaped points. The needles tend to form aggregates usually in the form of large irregular rosettes. Microscopically the crystals sometimes resemble maltosazone, but their melting-point is much lower, 152–153°. This osazone is soluble in hot water and in 20 % alcohol (Fig. 1).

*Osazone II. Glucosazone.* This is characterised by its fine long dark yellow needles which occur singly or more often in sheaves, brooms and rosettes. When formed from urine this osazone tends to float to the surface of the liquid forming a woolly mass in which crystals belonging to other types may be found entangled. If the amount is small, this osazone remains at the bottom among the deposit formed by the other crystals. When purified by solution in hot absolute alcohol and recrystallisation from dilute alcohol (about 20 %) this osazone melts at 202–204°, but when first obtained from urine its melting-point is much lower, 188–198°. The purified osazone is insoluble in hot water and in cold absolute alcohol but dissolves in the latter on heating. It appears to be identical with glucosazone in melting-point, colour, crystalline appearance and solubility. Fig. 7 represents a sheaf obtained from a normal urine voided 1½ hours after an ordinary meal.

The crystal mixtures have no fixed melting-point, solubility or crystalline form. These characteristics depend entirely on the amount contributed to the crystal by each osazone. This peculiarity might explain why so many investigators succeeded in obtaining from normal urines osazones with various melting-points and crystalline forms. But it is quite significant that the melting-point of all these new osazones lies between those of the two osazones already described. It is, therefore, highly probable that all such osazones are of the same nature as the crystal mixtures. The osazone isolated by Patterson [1926] seems to be a good example, for its crystalline form resembles closely certain crystal mixtures which can be easily prepared by dissolving a mixture of osazone I and osazone II in a little hot absolute alcohol and then fractionating it by simply diluting with water. As the alcohol becomes weaker, two to three well-formed precipitates come down. On examination the appearance and properties of these fractions will be found to be intermediate between those of the components.

The principal object of this investigation being to throw some light on the long-discussed question of the occurrence of glucose in normal urines, little attention has been given to the identity of osazone I. Baisch [1895], however, isolated from urines fermented with yeast an osazone which had m.p. 152–154°, and Lemaire [1895] later obtained a similar product of m.p. 150–151°. The former investigator believed his osazone to be identical with Fischer's *iso*-maltosazone both in crystalline appearance and melting-point, but apparently there has been no further evidence provided regarding the identity of Baisch's product. It is uncertain whether the product is really *iso*-maltosazone or not. The melting-point of the product isolated by Baisch and Lemaire agrees with

that given for osazone I, but the crystalline appearance of osazone I is totally different. For, while the osazone isolated by these observers crystallises in small rosettes composed of very fine needles, much finer than those of glucosazone, the needles of osazone I are broad, flat and large. This, however, may be due to differences in the method of preparation. An osazone prepared from a specimen of *iso*-maltose kindly supplied by Prof. Ling gave crystals similar to those described by Baisch and Lemaire, and when glucosazone was added to it and then recrystallised, crystal mixtures were obtained, but none of the crystals resembled osazone I.

Crystals similar to those described here for osazone I have also been obtained by Geelmuyden [1915] from normal urines and from urines of "sugar-free" diabetics.

*The detection of glucose added to ordinary urine.*

If glucose is added to ordinary urines the quantity that gives typical glucosazone will, as Höst [1923] pointed out, vary. Höst obtained typical glucosazones from ordinary urines after adding about 10 mg. glucose per 100 cc. and in some cases not before the addition of 20 mg. The causes of these variations may be appreciated from what has already been stated regarding interfering substances. There is little doubt that the tendency of the osazones of the urinary sugars to form crystal mixtures is responsible for these variations. Variable crystalline forms with mixed types of crystals are also obtained when aqueous solutions of mixed sugars are examined. From an aqueous solution containing glucose and lactose it sometimes happens that nothing but typical glucosazone is obtained. But this glucosazone though typical microscopically is a crystal mixture from which the lactosazone can be recovered by fractional crystallisation. This behaviour explains why the majority of diabetic urines give only glucosazone crystals, and also explains the disappearance of the "physiological osazone" when the amount of glucose added by Höst to ordinary urines was adequate to produce "typical" glucosazone. The melting-point of such a typical glucosazone obtained from urine is generally much lower than that of pure glucosazone, because of the other osazone incorporated in it. The elimination of the latter osazone, by fractional crystallisation, leads to a marked rise in the melting-point. In a particular case the melting-point went up from 188° to 202°, or just two degrees less than that of pure glucosazone. It is not always easy to separate osazone I in a pure state from this typical glucosazone, but this point may be said to have been satisfactorily proved, if the rise in the melting-point follows the elimination of an osazone whose characters are unmistakably different from those of pure glucosazone.

It appears, therefore, that the phenylhydrazine reaction is less sensitive for the detection of glucose added to ordinary urines. This is, however, not entirely correct for small quantities of added glucose may be detected by watching the minute changes in the crystal mixtures, as little as 2.5 mg.

glucose per 100 cc. producing a visible increase in the glucosazone element in some of the crystal mixtures.

*The phenylhydrazine reaction employed by Höst for the detection of glucose in normal urine.*

Höst [1923] employed a modified Neumann's phenylhydrazine reaction and no precautions were taken to remove interfering substances from the urine. The osazones obtained by Höst from normal urines differ enormously in appearance from those described here. When his method was applied to 14 urines, no glucosazone whatever was obtained, though two of these urines gave typical glucosazone by the method described here. Some of the "physiological osazones" described by Höst were subjected to fractional crystallisation and in many cases it was possible to split them into glucosazone and another osazone very similar to osazone I. It, therefore, seems that Höst's failure to obtain glucosazone from normal urine was not due to the absence of glucose but to the method employed.

*The occurrence of glucose in the urines of healthy individuals.*

The osazone test in the form described here was applied frequently to the urines of about 700 Egyptians. These were mostly students whose ages varied between 17 and 27 years. Every urine gave osazone crystals that differed in detail in different persons and even in the same person at different times. Great variety of crystal shapes was encountered but nevertheless they were all mixtures of the types already described. The frequency with which typical glucosazone crystals were obtained was rather striking. Of urines voided 4-5 hours after a mixed meal 12-15 % give typical glucosazone crystals as well as other crystals. The remaining urines give crystal mixtures in which the glucosazone element varies greatly. In urines voided 1-2 hours after an ordinary meal the percentage is practically doubled (20-30 %) and the crystal mixtures in all the urines show a definite increase in the glucosazone element over what is usually obtained 4-5 hours after a meal. After fasting for about 12 hours the urines of two out of 30 persons gave typical glucosazones.

The question now arises as to the state of the carbohydrate metabolism in those whose urine gives typical glucosazone. Two points should be grasped before this question is discussed. Urines giving typical glucosazone do not reduce Fehling's reagent or Benedict's reagent in the ordinary sense. Such urines may give a reaction similar to that given by normal concentrated urines if equal parts of the reagent and the urine are boiled for 20 seconds and allowed to stand for 10-30 minutes. Again every subject examined passed at times a urine that gave typical glucosazone. The relation of this to meals was not always clear for it happened even after a 12 hours' fast.

To clear up this point, the blood-sugar and the osazones of the urine-sugars were simultaneously studied in 28 student volunteers, just before and after giving each 50 g. pure glucose dissolved in 100 cc. water by the mouth after

fasting for about 12 hours. Samples of urine and blood were taken just before administering the glucose and at half hourly intervals afterwards. The fasting urine of two students gave typical glucosazone, all the others gave crystal mixtures. After the glucose none of the urines gave typical glucosazone even in the case of the two students whose fasting urine gave typical glucosazone. The behaviour of the blood-sugar was apparently quite normal, for the fasting levels varied from 0.078 to 0.120 g. % and the maximum from 0.120 to 0.198 g. % and the return to the fasting level occurred from  $1\frac{1}{2}$  to  $2\frac{1}{2}$  hours after taking the glucose.

It is justifiable to believe, then, that when urines give typical glucosazone the condition is not always due to an abnormal carbohydrate metabolism. This statement applies to specimens of urine taken at random during the day, for it has always been observed that a mixed 24 hours' urine of a healthy individual will only give crystal mixtures but no typical glucosazone. Under normal conditions the balance between the urine-sugars in a mixed 24 hours' urine appears to be such that glucose does not have a chance of forming typical glucosazone.

It is interesting to note that the excretion of glucose after 50 g. pure glucose seems to be less than that which follows an ordinary mixed meal.

#### DISCUSSION.

The failure of certain investigators, in particular Geelmuyden [1915] and Höst [1923], to obtain glucosazone from normal urines is probably due to the fact that the test is not sufficiently delicate to detect minute amounts of glucose unless certain precautions are observed. The most important of these is the removal by adsorption on charcoal of certain substances which interfere with the formation of the osazones. The tendency of different osazones to form crystal mixtures is another disturbing factor, which must be borne in mind when glucosazone is looked for. With the exception of crystals (*a*) and (*b*) in Geelmuyden's figure, and the few detached broad needles lower down in the diagram and also crystal (*m*), Geelmuyden's other crystals appear to be all of the mixed variety.

The results presented in this paper leave little doubt that glucose is a constituent of normal urine. Evidence for this can also be obtained from Höst's own results, for he observed that urines fermented with yeast required the addition of more glucose in order to give typical glucosazone crystals than unfermented urines. Höst himself suspected that this result might have the interpretation given to it here.

The increase in the reducing power of urines after ordinary meals seems to be due, at least in part, to an increased excretion of glucose and not, as has been suggested by Folin and Berglund [1922], to the presence of unassimilable carbohydrates and degradation products arising from carbohydrates during the preparation of the food. The paradoxical behaviour of the kidney towards glucose taken alone and that absorbed during the digestion of a mixed meal,

may have a great deal to do with the other digestion products absorbed with the glucose. Hamburger observed that changing the inorganic composition of a perfusate altered the permeability of the frog's kidney towards glucose.

#### SUMMARY.

1. A trustworthy method for the preparation of the osazones of the urine-sugars is described.

2. When this method is applied to aqueous solutions of glucose or to glucose dissolved in urines from which the normal sugars have been removed, glucosazone is obtained even when the concentration is as low as 1 mg. per 100 cc. In suitable cases glucose added to ordinary urines can be detected when the amount added is as low as 2.5 mg. per 100 cc.

3. Normal urines give numerous types of crystal mixtures, but these have been shown to be formed of a mixture of two simple osazones, one of which is identical with glucosazone and the other appears to correspond with Baisch's *iso*-maltosazone, as regards melting-point, but differs markedly in crystalline appearance.

4. The urines of over 700 persons have been examined under different conditions, and it has been found that typical glucosazone crystals are given by 20–30 % of the urines voided 1–2 hours after an ordinary meal; the percentage drops to 12–15 in urines passed 4–5 hours after meals, and it falls further to approximately 7 % after a 12 hours' fast. That this is, in most cases, not due to an abnormal carbohydrate metabolism, has been shown by examining the tolerance of 28 students to 50 g. glucose.

5. Evidence is given to show that some of Hüst's "physiological osazones" are impure crystal mixtures. It is also suggested that the same applies to some of Geelmuyden's osazones.

6. The old view that glucose in small quantities is a constituent of normal urine is confirmed.

I wish to thank Prof. J. C. Drummond for his kindness and advice. My thanks are also due to Dr F. N. Yassa and A. A. Hassan for helping me with the sugar tolerance tests.

#### REFERENCES.

- Baisch (1894). *Z. physiol. Chem.* **18**, 193.  
— (1895). *Z. physiol. Chem.* **20**, 249.  
Benedict, Osterberg and Neuwirth (1918). *J. Biol. Chem.* **34**, 217.  
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 213.  
Geelmuyden (1915). *Norsk Mag. Laegevidenskab*, **13**, 985.  
Greenwald, Gross and Samet (1925). *J. Biol. Chem.* **62**, 40.  
Hüst (1923). *J. Metab. Res.* **4**, 315.  
Lemaire (1895). *Z. physiol. Chem.* **21**, 442.  
v. Lipmann, quoted by Hüst (1923).  
Neuwirth (1922). *J. Biol. Chem.* **51**, 11.  
Patterson (1926). *Biochem. J.* **20**, 651.  
Favy and Siau (1901). *J. Physiol.* **26**, 282.  
Sumner (1921). *J. Biol. Chem.* **47**, 5.



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Fig. 1. Osazone I. m.p. 152-153 .  
Figs. 2-6. Crystal mixtures.  
Fig. 7. Glucosazone "impure."



# CLXIX. THE CENTENARY OF WÖHLER'S SYNTHESIS OF UREA (1828—1928).

By FREDERICK GOWLAND HOPKINS.

*Read at the Cambridge meeting of the Biochemical Society<sup>1</sup>,  
October 6th, 1928.*

CHARACTERISTIC of modern intellectual progress is the growth of the historic sense. Hence the popularity of commemorative celebrations, recalling not alone the outstanding events of political and social progress, but also those events which are landmarks in intellectual advance. This is surely a commendable form of piety, promoting in current thought a sense of continuity and a proper perspective.

In the history of Biochemistry the synthesis of Urea by Wöhler is certainly an outstanding event. Few who are concerned either with chemistry or biology can be unaware of the date of its accomplishment, for there is scarce a textbook that does not mention that date with insistence. A reminder therefore that the present year marks its centenary would be wholly superfluous. It has been felt however that in the records of the Biochemical Society there should be some indication that due notice was taken of the arrival of that centenary, and as a very inadequate method of marking such recognition I have been given the privilege on the present occasion of saying a few words about Wöhler's famous synthesis.

Now we should I think realise at the outset that in our commemorative piety we may be apt to overestimate the effect of this or that discovery upon the thought of its own period. In our backward perspective we see it stand out in all its importance, but it does not follow that this importance was immediately felt. I think, indeed, that our textbook writers in their familiar references to the event which we now have specially in mind have tended to exaggerate its effect upon the thought of its time.

The intrinsic historic importance of Wöhler's synthesis can hardly of course be exaggerated. So long as the belief held ground that substances formed in the plant or animal could never be made in the laboratory, there could be no encouragement for those who instinctively hoped that chemistry might join hands with biology. The very outermost defences of Vitalism seemed unassailable. We now recognise that Wöhler's discovery effectively breached these outworks, making it necessary for Vitalism to retreat behind inner and more subtle defences. The breach in question had to be widened, however, by later efforts, before very many recognised all its significance.

<sup>1</sup> Here slightly amplified.



Before proceeding, I ought perhaps to refer, parenthetically at least, to the work of the English chemist, Hennell, because his synthesis of ethyl alcohol has sometimes been put forward as being, rather than Wöhler's, the actual pioneer organic synthesis. The significant part of Hennell's work—his demonstration, namely, that the sulphovinic acid which (following Faraday) he had prepared from olefiant gas yielded alcohol on distillation—was as a matter of fact published by the Royal Society<sup>1</sup> in the same year as that in which Wöhler's paper appeared. We may well therefore hold the accomplishment of both syntheses in pious memory to-day. But with respect to their significance, it should I think be admitted that since the vital nature of alcoholic fermentation was not recognised in the day of Hennell and Wöhler, the artificial production of a substance actually arising in the living animal body should have had for contemporary thought the greater significance.

It is well, however, for the sake of historical accuracy, to realise that as I have already indicated this significance was not fully recognised when the work was first published. I may remind you that Wöhler himself in communicating his discovery<sup>2</sup> makes no resounding claims. He remarks merely, in the beginning of his paper, that the research he is to describe "gave the unexpected result that by the combination of cyanic acid with ammonia urea is formed; a fact (he continues) that is so far noteworthy in that it furnishes an example of the artificial production of an organic, indeed a so-called animal substance, from inorganic materials." The rest of this classical paper is wholly descriptive until, at its close, the author shows in a sentence that he is alert to the significance of the isomeric change which is involved in the synthesis. The adjective "noteworthy" (*merkwürdig*) is all that he allows himself in comment on his successful passage from the inorganic to the organic.

It is however not surprising that Wöhler when announcing his discovery wrote with this restraint, for it would seem that he himself was really doubtful whether he had, after all, built a bridge across a gulf. In a letter to Berzelius dated from Berlin, Feb. 22nd, 1828<sup>3</sup>, he writes as follows to his former chief: "You will perhaps remember that during a research carried out in those happy days when I still worked with you, I found that whenever one endeavoured to combine cyanic acid with ammonia, a crystalline substance appeared which was neutral, and gave the reactions neither of cyanic acid nor ammonia." This passage refers to work done in 1825. The letter I am quoting informs Berzelius, "for the first time," that the neutral substance in question was urea. "I must now tell you," writes Wöhler, "that I can make urea without calling on my kidneys, and indeed without the aid of any animal, be it man or dog." But at the end of a somewhat long letter he puts this question to his correspondent: "This artificial production of urea—can one take it as an example of the formation of an organic substance out of inorganic materials?"

<sup>1</sup> *Phil. Trans.* 1828, p. 365.

<sup>2</sup> *Poggendorff's Annal.* Bd. 12, 243 (1828).

<sup>3</sup> *Briefwechsel. Berzelius und Wöhler*, edited by O. Wallach, Bd. 1, 206 (1901).

It is striking that for the production of cyanic acid (and, no less, of ammonia) one must always start with an organic substance. The Natural Philosopher might well say that from animal carbon and from the cyanic compounds made from it the Organic has by no means disappeared, and it is for this reason that it may be always possible to reproduce an organic substance from these materials." An attitude sufficiently critical!

I cannot refrain from quoting the somewhat famous reply of Berzelius to this communication<sup>1</sup>, because it rather strikingly bears on my present theme—the effect of Wöhler's discovery upon contemporary minds. Berzelius wrote a letter which starts with sheer raillery of a Rabelaisian kind. In quoting it I propose to retain the language of a German translator! "Nachdem," says Wöhler's mentor, "Nachdem man seine Unsterblichkeit beim Urin angefangen hat, ist wohl aller Grund vorhanden, die Himmelfahrt in demselben Gegenstand zu vollenden—und wahrlich, Hr. Doktor hat wirklich die Kunst erfunden, den Richtweg zu einem unsterblichen Namen zu gehen.... Sollte es nun gelingen noch etwas weiter in Produktionsvermögen zu kommen (vesiculae seminales liegen ja weiter nach vorn als die Urinblase), welche herrliche Kunst in Laboratorium der Gewerbeschule ein noch so kleines Kind zu machen—Wer weiss? Es dürfte leicht genug gehen." Such was the first reaction of the great Swede's mind to Wöhler's announcement. Persiflage! Berzelius does not even trouble to answer the direct question posed for him. In the same letter he does, it is true, finally congratulate his former assistant upon a "right important and beautiful discovery," but it is noteworthy that his interest in it centred on the isomeric change involved in the synthesis. "It is an altogether striking circumstance," he remarks, "that the character of a salt is so completely absent (from the product) when the acid unites with ammonia, a circumstance which will certainly be very enlightening for future theories." It is clear indeed that Wöhler himself was on the whole more intrigued with this side of his discovery than with its bearings upon the vitalistic position. The method of urea formation suggested to his mind that alkaloids might somehow arise on similar lines from the union of organic acids with ammonia, and he made attempts to test this view, in the case of cinchonine for instance, by submitting the substance to electrolytic decomposition.

We should next, I think, take note of the attitude of Justus Liebig, who was in the closest touch with Wöhler and whose influence upon the thought of the time was great. Considering how much Liebig's own work contributed to the growth of clear thought and objective views in the domain of plant and animal chemistry, it is puzzling that he himself should so long have clung to his conception of a Vital Force. He does not, it is true, seem to have thought about it as a "Force" necessarily outside the category of known "forces." Living to-day he would probably have spoken of it as an unknown form of energy. It was for him an agency which reacted with (and in general opposed!) the forces of chemical affinity as understood by him. Even so late as 1842 he

<sup>1</sup> *Briefwechsel, Berzelius und Wöhler*, edited by O. Wallach, Bd. 1, 208 (1901).

wrote: "The vital force appears as a moving force or cause of motion when it overcomes the chemical forces (cohesion and affinity) which act between the constituents of food, and when it changes the position or place in which their elements occur; it is manifested as a cause of motion in overcoming the chemical attraction of the constituents of food and is further the cause which compels them to combine in a new arrangement, and to assume new forms<sup>1</sup>."

This was written fourteen years after the synthesis of urea, with which of course Liebig was from the first intimately familiar. That accomplishment clearly did not convince him that the forces which "compelled" the elements to "combine in a new arrangement" in Wöhler's laboratory could do the same in the living body. It is but fair to remember that some years later Liebig was coming to the conclusion that it was after all unjustifiable to assume the existence of a special vital force until more was learnt concerning the scope of known physical forces. To anyone who reads the 20th letter in the 5th edition (1851) of his *Chemische Briefe* this change of mind will be evident. By this time however Wöhler's synthesis had become an old story.

Great as is the debt of Biochemistry to Liebig, there can be no doubt that he lacked physiological knowledge and his *ex cathedra* utterances sometimes tended to obscure the facts. Berzelius insisted on this and spoke of current theories based solely upon chemical speculations, untested on plant or animal, as "Probabilitätsphysiologie"!

I must not here leave you with the impression that Liebig's obsession was shared by all the leading minds of that day. Dumas, for instance, though later a supporter of many of Liebig's much disputed views about other aspects of biochemical phenomena, early dissociated himself from a belief in any Vital Force. It may be noted that in 1833 Dumas met Wöhler for the first time. This was in Paris and the two evidently saw a good deal of each other there, for Wöhler sent to Berzelius<sup>2</sup> an extremely acute appraisal of the character and qualifications of the French chemist. Three years later, in 1836<sup>3</sup>, Dumas allowed himself the following very definite statement of faith—"In my belief there are no substances which, in any peculiar sense, are to be called organic. I visualise in organised beings only very slowly working forms of apparatus which act upon materials at the moment of their genesis and thus produce from a few elements very various 'inorganic' compounds." (The quotation marks to the word 'inorganic' are mine.) This statement has a very modern ring; I am even inclined to think that it contains a hint for ourselves. It is impossible to say how far the synthesis of urea had affected the thought of Dumas; he was certainly familiar with it.

In seeking evidence concerning the contemporary effect of Wöhler's work we may perhaps do well to turn from the minds of the Olympians and ask how it affected lesser men; the men who in the main were concerned with teaching

<sup>1</sup> *Animal Chemistry*, edited by Wm Gregory from the author's ms., 2nd ed. 1843, p. 204.

<sup>2</sup> *Briefwechsel*, Bd. 1, 535.

<sup>3</sup> *J. prakt. Chem.* 7, 298 (1836).

and the writing of students' textbooks. They were little concerned with philosophical niceties, but any considerable change in the intellectual atmosphere must have affected their writings.

The chemical textbooks published during the decade which followed Wöhler's publication, while duly chronicling the success of the synthesis and its nature, seem to make little of its anti-vitalistic significance, even in the case of those specially concerned with what we should now call biochemistry. A representative book of the kind was the *Medical Chemistry* of Carl Fromherz, of which the first volume appeared in 1832 and the second in 1836. Fromherz was professor of Chemistry at Freiburg and an able writer. His book was one of the best of its time and I think it is worth while to note the attitude of this author to the event which is occupying our attention. Fromherz, in a section of his book devoted especially to urea, describes Wöhler's synthesis in detail, remarking with restraint that it is an accomplishment of much interest. But in the theoretical discussion contained in the book, when dealing with what he believed to be the fundamental distinction between inorganic and organic chemistry, he frankly resigns himself to a belief in a vital force, and shows no signs of feeling that Wöhler's work ought to affect that belief in the least. There were at this time several other writers who though far from being obscurantists, continued to believe in a Vital Force; though among them were some who, like Fromherz, had the sincerest belief in the importance of chemical studies to biology. The men I mean thought on chemical lines and it was for chemical reasons (as they would themselves have claimed) and not because of philosophical preconceptions that for some years after Wöhler's synthesis they relied upon something unique as the causal factor in biochemical phenomena.

Their attitude was due in fact to the dominance in contemporary thought of that rigid dualistic theory of chemical affinity which we associate especially with the name of Berzelius.

Inorganic compounds arise, it was held, only when elements of opposite electric charges—or groups dominated by elements with opposite electric charges—come together. Now the writers I am quoting found it difficult to see how in the building up of the complex carbon compounds, produced by the plant or animal, this rigid duality could possibly play an effective part, and their doubts expressed more wisdom perhaps than Berzelius himself showed in his struggles to apply his views in organic chemistry.

I am dealing with the matter far too briefly to make clear the weight of such considerations as judged from the standpoint of the time; but to Fromherz and many of his contemporaries it was plain that, while inorganic compounds arose under the influence of affinity, as then understood, another, and quite different, force must control the knitting together of complex organic compounds. For these plain chemical thinkers the Vital Force was just this unknown controlling agency. They had no other commitments with regard to it and were much less dogmatic than was Liebig in his earlier days.

You will see that Wöhler's synthesis was not one to disturb very greatly such views as those of Fromherz. The union of ammonia and cyanic acid is a straightforward dualistic happening, and the isomeric change involved—though Wöhler himself was alert to its general significance—was still merely obscure to the majority.

In parenthesis, I may say here that I cannot find that the first artificial synthesis of an organic substance formed a text for discussion in any of the Journals of Wöhler's time devoted to philosophical or general intellectual interests; though a complete research into this matter has been beyond my leisure. One gets the impression that the intelligent lay public which took cognizance of scientific progress in those days was hardly aware that anything worthy of its attention had yet arisen from the application of chemistry to the plant or animal.

Returning to the textbook writers of the time, one feels that it was a sound instinct in these pioneers to emphasise in their own way that there is all the difference between the methods of the living body and the classical methods of the laboratory. That the agencies which control biochemical events work on lines of their own is the basic circumstance which alone justifies the claim of biochemistry to rank as a self-standing scientific discipline. It is those special agencies and their impact upon molecular structures which constitute the peculiar field of biochemical study, and should be its final concern. The information which artificial syntheses afford in proof of constitution is of course absolutely essential to the progress of biochemistry, but for the genuine biochemist its attainment is a means to an end and not an end in itself. We can never be content with what Berzelius would have called "Probabilitätsbiochemie."

Fortunately we are no longer compelled to visualise an ultra-physical Force as the determinant of chemical changes in the living cell. Just now Surface Catalysis as an agency justifiably dominates our field of view, though it may be doubted whether it alone will give us the complete picture we hope ultimately to see. It is, I think, for the progress of biochemistry of good augury that the organic chemist now views molecular structure as a dynamic and not a static affair, for it is sure that molecules, while playing their diverse parts in the intense turmoil of life, may be never more than momentarily in that stable condition which they have assumed when they get into bottles on our shelves.

It is certainly instructive and not necessarily humiliating to realise that the molecular structure of a substance already synthesised one hundred years ago, a substance with a molecular weight of no more than 60, is yet a matter for debate. It is not surprising. A molecule of such biochemical prominence is likely to assume during its complex metabolic career just as many forms as with due regard to the decencies of valency it can manage to assume! I think we should be grateful to Prof. Werner for the "ring" and "open" structural formulae of urea, even if their reality be not yet proven. I also

think, however, that carbamide may not be absent from the laboratory of living tissues. After all, ammonia *does* meet with carbon dioxide there, and the liver *can* dehydrolise ammonium carbonate. Anyhow, it is significant that a century after Wöhler's synthesis we are still discussing this matter, and are realising in consequence that we must revise our too dogmatic views as to precisely how urea arises from amino-acids in the body.

It would indeed not be out of place when celebrating the centenary of its synthesis to recall how interesting are the problems which, in all its relations, this familiar substance still presents. I must not of course stop to survey them.

Nevertheless, just to widen a little the scope of my remarks, I will venture in concluding them to put before you certain considerations of a more biological sort which seem to me to possess no small interest. On surveying the field of comparative biochemistry we remember that urea, with all its obvious merits as an excretive, is in the case of certain animal groups replaced by insoluble uric acid. The replacement at first sight seems a casual happening and has puzzled many. My colleague, Dr Joseph Needham, has been considering this problem and has come to a conclusion which with his permission I will submit to your judgment. The evidence on which it is based will be found in an article which Needham is publishing in *Science Progress*, and later, in a chapter of his forthcoming book on the Chemistry of Development.

The thesis is that the conditions of its embryonic existence determine whether an animal shall excrete urea or uric acid. If its embryonic development occurs in an egg subject to aquatic conditions which allow the end-products of metabolism to diffuse freely away, or if, as in the case of the viviparous mammal, the maternal circulation secures the same efficient removal, then the embryo, and, by established metabolic habit, also the adult, excretes urea. If on the other hand the embryonic life is passed in a closed box, such as is the bird's egg, then the embryo, for good reasons, as we are to see, turns to uric acid as an end-product and excretive, and the adult continues the tradition.

Consider the state of affairs in the hen's egg. Up to the eleventh day water accumulates in the allantois in sufficient amount to transport the uric acid produced up to that date. Then water is re-absorbed from the allantoic cavity leaving uric acid behind. Later water is again excreted, carrying more uric acid. "It is," to use Needham's words, "as if the water acted as an endless belt-conveyor transferring uric acid from the cells of the embryo into the allantoic liquid and then returning to convey more." Thus the embryo itself escapes any accumulation of its excretion. On the other hand it is hard to see how embryonic development in the closed system of the bird's egg could proceed satisfactorily if the end-product of its metabolism were a very soluble and highly diffusible substance like urea. It would accumulate in equal concentrations throughout the contents of the egg, and the embryo itself would, as Needham remarks, become more and more uraemic! Under these conditions the less soluble excretion possesses all the advantages. Needham

has brought together all the available data concerning the excretion of urea and uric acid respectively in the various animal phyla, and the weight of the evidence strongly supports the view that embryonic conditions determine the nature of the end-product. That the adult continues to behave on the same lines as the embryo merely illustrates once more the fact that every organism is a product of its past. Such considerations are doubtless of the teleological kind, but they help us to understand an evolutionary puzzle.

Perhaps I have said too little about Wöhler's synthesis and too much about other matters, and what I have said must seem to have minimised rather than emphasised the importance of its influence upon thought. Yet its intrinsic significance is of the greatest. Not, it is true, until the structure of biochemical science had grown greater than it was in the days of Wöhler and his immediate contemporaries was it recognised as one of the foundation stones of that structure; but as such we now see it.

# CLXX. DYNAMICS OF THE WAXY GENE IN MAIZE.

## II. THE NATURE OF WAXY STARCH<sup>1</sup>.

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*(Received July 18th, 1928.)*

### INTRODUCTION.

THE waxy factor in maize occasions a conspicuous change in the character of the reserve starch. In analysing the physiological action of the gene we have sought to determine how this storage product differs from the starch laid down in the endosperm and pollen of non-waxy maize. Two questions of primary importance are presented. Are the waxy and non-waxy starches composed of the same ultimate sugar units? And, secondly, are the sugar residues united in the same fashion in the respective elementary starch molecules? Perhaps a third query might be added with reference to degree of association.

A mode of attack somewhat different from that formerly employed [Brink and Abegg, 1926] has been adopted in the present investigations. Malt amylase has been used as the exploring agent with a view of ascertaining the particular sugar or sugars formed on hydrolysis of the starch. It is by this means that an answer to the first question set forth above has been sought. The rate of conversion of waxy starch to sugar under the action of malt amylase has likewise been carefully investigated for the light it might throw upon the structure of the starch molecule. The phosphorus content of the starch has been determined and the relative amounts of the two main constituents of the starch granule, namely the  $\alpha$ -amylose and  $\beta$ -amylose of Meyer's [1895] terminology, have been reinvestigated by the barley diastase method [Ling and Nanji, 1923].

The chemistry of the polysaccharides has not yet advanced to a stage where the structural formula of starch can be set down. This fact imposes close limitations upon an investigation of the present type. Perhaps the time is yet distant when we shall be able to describe the effects of such a gene in maize as waxy in terms of changes in molecular structure. Even though the chemical foundation upon which such investigations might be built is incomplete it seems worth while, in view of the importance of the subject, to study the dynamic properties of the hereditary units by such methods as are available.

<sup>1</sup> Papers from the Department of Genetics, Agricultural Experiment Station, University of Wisconsin, No. 88. Published with the approval of the Director of the Station.



*Proportions of  $\alpha$ - and  $\beta$ -amylose by the barley diastase method.*

According to Ling and Nanji [1923] precipitated barley amylase added to a starch paste at 50° converts the  $\beta$ -amylose (amylose) to maltose while the  $\alpha$ -amylose (amylopectin) is scarcely attacked. These workers adopted this procedure in separating the two principal constituents of the starch grain.

In preparing the enzyme 200 g. ground barley are suspended in 600 cc. water and a few drops of toluene added as an antiseptic. The suspension is allowed to stand overnight at room temperature. The liquid is then removed in a lever press, filtered, and treated with three volumes of 95 % alcohol. The rather copious precipitate formed, containing the barley amylase, is promptly washed with absolute alcohol and redissolved in water.

The content of  $\beta$ -amylose was determined on waxy maize starch prepared in the laboratory [Brink and Abegg, 1927], and on a high grade commercial sample of common maize starch. 5 g. samples were gelatinised in boiling water, transferred to flasks and made up to 250 cc. after cooling. The  $p_H$  was adjusted to approximately 5.0 through the addition of dilute acetic acid. For the conversion 7 cc. of the barley diastase solution (equivalent to about 2 g. barley) and two drops of toluene were added to each sample. The flasks were corked and placed in a water-bath at 50°. After 18.5 hours the conversion was stopped by boiling. The solutions were cooled and filtered and the "total solids in solution" in the filtrate determined by the specific gravity method [Brown, 1904]. The amounts of maltose formed were measured iodimetrically according to the procedure outlined by Baker and Hulton [1920]. The proportion of starch converted was then directly computed from the maltose values.

Duplicate determinations gave 48 and 47 % respectively of the starch converted in the case of the common sample. One lot of the waxy starch was accidentally lost, the other showed 49 % of the starch converted. The barley diastase method, therefore, reveals little difference in the content of  $\beta$ -amylose in common and waxy starch. In both there would appear to be approximately equal amounts of  $\alpha$ - and  $\beta$ -amylose.

It will be recalled that the centrifugation method of Sherman and Baker [1916] gave 8 % of  $\beta$ -amylose in common maize starch and about 60 % in waxy starch [Brink and Abegg, 1926]. Evidently different things are being measured by the two methods. The biochemical test indicates no important difference in the relative amounts of  $\alpha$ - and  $\beta$ -amylose; but the two substances may occur in quite different physical conditions in the two kinds of starch pastes giving quite different results on centrifugation.

*Hemicellulose in waxy and non-waxy starch.*

When common maize starch paste is treated at 50° with malt diastase there appears, in 10 to 30 hours, depending upon the enzyme concentration, a rather copious flocculent precipitate. Schryver and Thomas [1923] found that this material gives a blue coloration with iodine but resists digestion with

taka-diaztase. They conclude that the substance is a hemicellulose. It is not known whether the hemicellulose is an integral part of the starch grain or whether it is present as an impurity. The former seems more probable. Owing to the rather small supply of laboratory-prepared waxy starch at hand a quantitative estimation of hemicellulose content was not made. It was evident, however, from the relatively clear appearance of the waxy starch conversion liquids that practically no hemicellulose was present. Common maize starch, on the other hand, contains significant amounts of it. Schryver and Thomas [1923] report 3.6 %.

The presence of hemicellulose in starch introduces difficulties in measuring rate of conversion by noting the time required for the solution to lose the power of giving a colour reaction with iodine. Our first experiments [Brink and Abegg, 1926], in which this method was employed, afforded results which led to the conclusion that waxy starch is more readily hydrolysed than the common sort. The difference was apparent when 1 % HCl was used as the hydrolysing agent but it seemed particularly large when salivary amylase or the amylases from waxy and non-waxy maize seedlings were employed. The procedure used, however, has been shown to be inadequate. If starch were the only substance present giving the colour reaction the method would be valid but, as noted above, Schryver and Thomas [1923] have shown that maize starch contains hemicellulose which reacts with iodine but is resistant to amylase. Owing to the hemicellulose present, colour may be given by the conversion liquid long after the starch has been hydrolysed.

In the present studies the behaviour of the waxy and non-waxy starches on treatment with malt amylase has been carefully investigated and our former conclusions on comparative rates of hydrolysis are revised.

*The sugar formed on treating waxy and common maize starches with amylase.*

The view is commonly held that under the action of amylase starch is split quantitatively into a single sugar, maltose. A number of investigators, however, dissent from this conclusion. Pringsheim [1923] contends that adequate proof of the formation of but one sugar has not been furnished and, according to Armstrong [1924, p. 164] "There is little doubt that the starch molecule contains glucose as well as maltose residues." Ling and Nanji [1923] hold that, on hydrolysis with malt amylase,  $\beta$ -amylose (amylose) forms maltose, and  $\alpha$ -amylose (amylopectin) produces two sugars, maltose and isomaltose. The weight of evidence, nevertheless, seems to be in favour of the contention that starch is composed entirely of maltose residues [Maquenne and Roux, 1905].

We have sought to determine whether the reserve starches of the waxy and non-waxy races of maize differ with respect to the sugar formed on diastatic hydrolysis. The conversions were carried out in two stages. The starch paste was first treated with alcohol-precipitated barley diastase which hydrolyses the  $\beta$ -amylose and scarcely changes the  $\alpha$ -amylose. The latter substance was

then separated from the other products of the first conversion and hydrolysed with an active preparation of malt amylase (D.P. = 170°)<sup>1</sup>. Reducing power, specific rotation and the osazone test were employed in identifying the sugars in the respective conversion liquids.

*β-Amylose fraction of common maize starch.*

40 g. of a high grade commercial sample of common maize starch were gelatinised by boiling and made up to 800 cc. 0.4 g. of barley diastase was added and the conversion allowed to proceed on a water-bath at about 50°. After 24 hours hydrolysis was stopped by boiling. The liquid was evaporated to a small volume and poured into alcohol giving a final concentration of 60 %. The precipitated α-amylase was filtered off and set aside for further treatment.

The filtrate containing the conversion products of the β-amylase was concentrated to a small volume and poured into an amount of alcohol giving a final concentration of 90 %. From the resulting precipitate the sugars were taken up by boiling, leaving a light, sticky precipitate, which was discarded. The supernatant liquid was filtered and the alcohol removed by heating on a steam-bath. The alcohol-free syrup from the β-amylase portion of the starch was taken up with water, a little toluene added as a preservative, and allowed to stand for two days in a corked bottle.

During the period of standing a light, brownish precipitate formed. This was filtered off leaving a fairly clear but yellowish solution. The concentration or "total solids in solution" was found by the specific gravity method to be 7.25 %. The reducing power was measured iodimetrically and the specific rotatory power determined. These constants were as follows<sup>2</sup>:  $R = 93$  and  $[\alpha]_{D_{4.0}} = +144.6^\circ$ . The corresponding values for maltose are  $R = 100$  and  $[\alpha]_{D_{4.0}} = +138^\circ$ . It seems probable from these results that some material of a dextrinous nature was still present as an impurity in the solution after the second treatment with alcohol. The formation of a precipitate on standing also bears out this contention. Dextrins would tend to lower the reducing power and increase the specific rotation. When the osazone was prepared definite evidence for the presence of maltose was obtained. A trace of glucosazone was also observed but no crystals of the isomaltosazone type [Ling and Nanji, 1923] were present.

It is concluded that the sugar formed from the β-amylase portion of common starch is maltose. Very small amounts of glucose may also occur.

<sup>1</sup> The diastatic power (D.P.) is taken as 100 when 3 cc. of a solution containing 0.1 g. in 250 cc. (1.2 mg.) of diastase produce sufficient maltose in 1 hour at 70° F. to reduce 5 cc. of Fehling's solution.

<sup>2</sup>  $R$  = reducing power. Measured in terms of an equivalent amount of maltose taken as 100.

The subscript to  $[\alpha]_{D_{4.0}}$  in this case 4.0, denotes the solution factor used in computing the concentration from the observed specific gravity.

*$\alpha$ -Amylose fraction of common maize starch.*

The  $\alpha$ -amylose portion precipitated in 60 % alcohol was taken up with sufficient water to give a fairly limpid solution and reprecipitated in 60 % alcohol. The filtrate was discarded. After kneading out the excess liquid the  $\alpha$ -amylose was obtained as a firm, non-elastic, non-adhesive, white mass, sparingly soluble in water. A portion of the solution was filtered by suction. The constants on this were:  $R = 13.2$  and  $[\alpha]_{D_{40}} = + 255^\circ$ .

The remainder of the  $\alpha$ -amylose was made up to a volume of 300 cc. with water and 0.2 g. malt amylase (D.P. 170) was added to it. It was placed on a water-bath at  $50^\circ$  for 48 hours. The solution was then cooled, and the hemi-cellulose filtered off. The clear solution was returned to the bath and a further 0.2 g. malt amylase added. Digestion was continued for another 48 hours.

At 96 hours a 50 cc. sample was taken, heated to boiling to inactivate the amylase, cooled and filtered. The solution was made up to 100 cc. and found to contain 0.76 % solids. The constants were:  $R = 104.6$  and  $[\alpha]_D = + 138.1^\circ$ . Several osazone preparations were made. In recrystallising these it was invariably found that an insoluble residue was present. No precipitate formed during heating. The recrystallised material showed maltosazone and a small amount of glucosazone. *iso*Maltosazone did not appear to be present.

The evidence indicates that the principal sugar present was maltose. Some glucose also occurred and a small amount of dextrin. The difficulties in recrystallisation were probably due to the latter substance.

 *$\beta$ -Amylose fraction of waxy maize starch.*

An examination of the sugars formed on the diastatic hydrolysis of waxy starch was carried out in the same way as with the common maize starch. 50 g. of waxy starch were gelatinised in hot water and the suspension made up to 1000 cc. 0.6 g. of alcohol-precipitated and dried barley diastase was added. The mixture was placed on a water-bath, at about  $50^\circ$ , and frequently shaken.

The digestion was stopped after 19 hours by boiling. The liquid was cooled and filtered and, after evaporating to 300 cc., poured into a volume of alcohol giving a final concentration of 60 %. The  $\alpha$ -amylose was precipitated as an elastic mass. Suction was used in filtering and the free liquid pressed out of the  $\alpha$ -amylose mass by kneading.

The filtrate was milky in appearance and formed a precipitate on standing. This was filtered off and the clear filtrate evaporated to 140 cc. The concentrated liquid was poured into alcohol, giving a final concentration of about 80 %. A further light precipitate formed which did not dissolve on boiling. The solution was cooled and filtered. The precipitate gave a distinctly violet reaction with iodine. The filtrate was concentrated to a thick syrup on the water-bath and allowed to stand at room temperature overnight. It did not crystallise. The syrup was then diluted to 250 cc. and the constants determined on the solution. These proved to be as follows:  $R = 96.3$  and  $[\alpha]_D = + 132.5^\circ$ .

A number of osazones which were prepared showed well-defined crystals of maltosazone and some glucosazone. A few crystals resembling those which Ling and Nanji considered to be isomaltosazone were also observed, but these did not occur consistently. These results indicate that the waxy conversion liquid contained principally maltose, together with small amounts of glucose and dextrans. The latter substances, not entirely removed by the treatment with alcohol, account for the fact that the observed reducing power is below 100. The rotatory power of the liquid is below that expected for a solution of pure maltose ( $[\alpha]_D = +138^\circ$ ). Dextrans would tend to make the value higher. Glucose, on the other hand, would depress it, and the observed deviation is probably due to the combined effect of the glucose and dextrin impurities.

*$\alpha$ -Amylose fraction of waxy maize starch.*

The  $\alpha$ -amylose separated from the barley diastase conversion liquid by precipitation with alcohol is obtained as an amorphous mass quite different in texture from the corresponding fraction of common maize starch. The latter is firm and crumbly and difficultly soluble in water, whereas the waxy  $\alpha$ -amylose is elastic, sticky and easily dissolved in water at ordinary temperatures.

The crude waxy  $\alpha$ -amylose obtained in the initial precipitation with 60 % alcohol was dissolved in water and reprecipitated with 60 % alcohol. After filtering, the  $\alpha$ -amylose was freed from alcohol by dissolving in water and concentrating on a steam-bath. A small portion of the material was taken for determination of the reducing power and the specific rotation. The constants were found to be as follows:  $R = 12.6$  and  $[\alpha]_D = +167^\circ$ . It will be recalled that the corresponding values for the  $\alpha$ -amylose from common maize starch were  $R = 13.2$  and  $[\alpha]_D = +225$ . It may be remarked here that while the specific rotations in these two cases may be widely different, great emphasis is not to be put upon the magnitude of the values actually observed. In the case of the common  $\alpha$ -amylose in particular the substance is imperfectly dissolved and the methods of clearing and filtering appreciably influence the rotatory power of the solution. Moreover the optical behaviour changes on standing. The sugars do not present these difficulties, but our experience with the more complex carbohydrates agrees with that of others in that comparable readings are very difficult to obtain.

A small portion of the waxy  $\alpha$ -amylose was treated with an active culture of beer yeast and the accessory conditions were made favourable for fermentation. No fermentation ensued, however, showing that neither maltose nor glucose was present. The reducing power of the  $\alpha$ -amylose preparation is probably due to the liberation of reducing groups in the substance itself by the alkaline iodine solution.

The remaining portion of the  $\alpha$ -amylose was dissolved in water and treated with 0.2 g. of malt amylase (D.P. 170) at  $50^\circ$ . After 24 hours the liquid was cooled and a very light precipitate filtered off. A further 0.2 g. of malt amylase (D.P. 170) was then added and the material again digested at  $50^\circ$ .

At 90 hours the reaction was stopped by boiling. A light precipitate was filtered off after cooling. The constants were:  $R = 100.2$  and  $[\alpha]_D = +133^\circ$ . After treatment with alcohol giving a final concentration of 90 % the conversion liquid showed  $R = 100.9$  and  $[\alpha]_D = +136^\circ$ .

The osazone test gave crystals rather ill-defined in appearance. Some of the crystals directly obtained simulated those of the isomaltosazone of Ling and Nanji [1923]. After recrystallisation, however, well-defined maltosazone crystals were formed. In these preparations no other types of crystals were present. The difficulties in obtaining a satisfactory test without recrystallisation were probably due to small amounts of dextrans present as impurities.

A sample of the conversion liquid from the  $\alpha$ -amylose portion of waxy starch was inoculated with beer yeast and a little peptone added. After a few hours' incubation a vigorous fermentation developed. According to Ling and Nanji [1923] isomaltose is not fermentable with yeast, whereas maltose, as is well known, is readily attacked.

The experiments on amylase hydrolysis may be summarised as follows. The gelatinised starches of both waxy and common maize form maltose when treated with the amylase of ungerminated barley. Presumably only the  $\beta$ -amylose portion of the starch is attacked by this enzyme. Small amounts of glucose are also formed in both cases. The  $\alpha$ -amylose residues of both kinds of starch when treated with malt amylase form maltose. Again traces of glucose may be found in the conversion liquids and small amounts of dextrin-like substances are present. These experiments afford no evidence for the existence of isomaltose among the diastatic conversion products of starch. They rather indicate that starch is split into maltose, the yield of this sugar closely approaching the theoretical.

From the genetic point of view the conclusion of particular interest to which these results point is that the two kinds of starches, waxy and non-waxy, are alike with respect to the ultimate sugar unit of which they are composed.

#### *Rate of hydrolysis with malt amylase.*

The foregoing studies indicate that waxy and common maize starches are composed of the same sugar residue, for both produce maltose on diastatic hydrolysis. Perhaps the essential difference between the carbohydrates lies in the manner in which the sugar molecules are united, that is, in the structure of the starch constituent molecules. If this is the case, one might expect that on treatment with a given amylase preparation the two starches would be hydrolysed at different rates.

Nine tests have been made on the rate of maltose formation from waxy and non-waxy starch pastes treated with a common enzyme under closely comparable conditions. The results are presented in Tables I and II. In tests 1 and 2 waxy starch prepared in the laboratory was compared with a high grade commercial sample. In the remaining experiments both the waxy and common maize starches used were separated from the two respective classes

of seed on segregating ears and purified by ourselves. The method employed in preparing these starches [Brink and Abegg, 1927] yields a product of very high purity.

Except in test 1, where an enzyme preparation of relatively low diastatic power was employed, one lot of malt amylase was used throughout. This was prepared from malt extract by diluting the latter with water to one-fifth and then treating it with three volumes of 95 % alcohol. The precipitated amylase was dried with absolute alcohol and stored in powder form. This preparation, which was used in several of the experiments discussed earlier, showed a diastatic power (D.P.) of 170 on the Lintner scale. This is a relatively high activity.

The starches were gelatinised in hot water and brought to volume after cooling. The acidity was adjusted with dilute acetic acid to approximately  $p_H$  4.5. The amylase was weighed out and dissolved in water and the required amounts were measured with a pipette. In each trial the digestions of the waxy and non-waxy starches were carried out side by side in the same water-bath.

At the intervals shown in the tables, 50 cc. samples of the conversion liquids were taken for maltose determinations. The amylase was inactivated by boiling. The reducing power was determined by the iodimetric method of Baker and Hulton [1920].

In computing the results the amount of maltose found is expressed as a percentage of the amount of moisture-free starch initially present. In test 5 the maltose values are reported in terms of the "total solids in solution" computed from the specific gravity of the liquid by means of a solution factor. The two methods give values which are closely alike after about 1.5 hours' digestion but prior to this the gravity basis yields higher percentages.

The conditions under which several conversion tests were carried out differ particularly with respect to concentration of amylase and the temperature as follows.

Test 1. 5.0 % starch pastes; 0.3 g. malt diastase of D.P. 50 in 1000 cc.; temp. ca. 50°.

Test 2. 4.0 % starch pastes; 0.03 g. malt diastase of D.P. 170 in 800 cc.; temp. 35°.

Test 3. 4.0 % starch pastes; 0.01 g. malt diastase of D.P. 170 in 600 cc.; temp. 35°.

Test 4. Same as test 3.

Test 5. 4.0 % common starch paste, 4.2 % waxy starch paste; 0.2 g. malt diastase of D.P. 170 in 700 cc.; temp. 50°.

Test 6. 4.0 % starch pastes; 0.005 g. malt diastase of D.P. 170 in 350 cc.; temp. 35°.

Test 7. 4.0 % starch pastes; 0.01 g. malt diastase of D.P. 170 in 350 cc.; temp. 35°.

Test 8. Same as test 7.

Test 9. Same as test 7.

Table I. *Maltose (%) reckoned on moisture-free starch.*

Time (hours) ...	$\frac{1}{2}$	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	3	5	8	25
Test 1 Common starch	33.8	43.6	47.9	51.4	51.1	53.9	56.5	56.2	62.0
Waxy starch	37.7	44.1	45.7	47.6	47.8	47.8	49.9	51.0	54.4
Time (minutes) ...	10	20	30	50	70	90	120	180	
Test 2 Common starch	13.0	19.7	27.5	39.3	48.1	50.2	51.1	67.2	
Waxy starch	12.5	14.6	19.0	28.7	30.8	36.3	40.5	54.5	
Time (minutes) ...	15		30		90		180		270
Test 3 Common starch	22.5		33.5		47.5		63.5		71.0
Waxy starch	21.0		33.5		47.5		66.0		71.5
Test 4 Common starch	19.0		29.0		47.5		64.5		73.0
Waxy starch	18.5		26.5		48.0		64.5		69.5
Time (minutes) ...	15		45			120		210	
Test 6 Common starch	5.7		16.2			32.9		42.6	
Waxy starch	7.4		15.0			25.3		26.7	
Time (minutes) ...	15		45			90		180	
Test 7 Common starch	13.2		28.4			45.6		59.2	
Waxy starch	13.2		22.8			32.7		42.8	
Test 8 Common starch	20.9		36.9			52.8		63.3	
Waxy starch	18.4		34.5			48.3		58.1	
Test 9 Common starch	14.9		29.9			46.4		61.2	
Waxy starch	11.9		28.5			42.4		54.2	
Aver. Common starch	16.3		31.7			48.3		61.2	
Waxy starch	14.5		28.6			41.1		51.7	

Table II. *Maltose (%) reckoned on moisture-free starch.*

Time (hours) ...	$\frac{1}{2}$	1	3	6	10
Test 5 Common starch	76.4	80.6	86.4	89.0	92.5
Waxy starch	70.6	76.0	79.3	84.1	88.1

In seven of the nine comparative tests maltose is formed more rapidly from the common starch than from the waxy starch. In the two remaining trials, tests 3 and 4, there is no certain difference in either direction. In test 1 the initial waxy samples gave higher percentages of maltose but the subsequent tests do not bear out this finding. Analysis of conversions attenuated through using small amounts of enzyme and a temperature of 35° shows that from the beginning the non-waxy starch is hydrolysed more rapidly.

In test 5 where conditions were favourable for rapid conversion the difference in rate in favour of the common starch is maintained to the stage where the digestion is about 90 % complete.

Tests 7, 8 and 9 were carried out with the greatest degree of refinement and the respective average percentages of maltose are plotted in Fig. 1.

The weight of evidence clearly favours the conclusion that malt amylase converts the common maize starch to maltose more rapidly than it does waxy starch. This is an important result for it gives some basis for thinking that there is a fundamental structural difference in the molecules of waxy and non-waxy starch.



*Intermediate products in amylase hydrolysis.*

It is a matter of interest whether the same intermediate compounds between starch and maltose are formed when the waxy and non-waxy reserve carbohydrates undergo diastatic hydrolysis. Some light is thrown on this question by observations on the optical activity of the conversion liquids during the digestion. In test 5 (Table II) such observations were made and were computed. In Fig. 2 the specific rotations are plotted against the percentages of maltose.

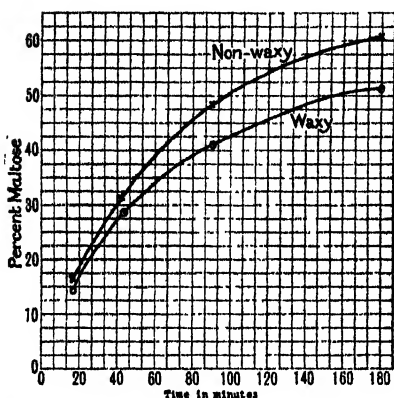


Fig. 1. Rate of maltose formation from the waxy and non-waxy starches when treated with a common enzyme, malt amylase. Average of results in tests 7, 8 and 9.

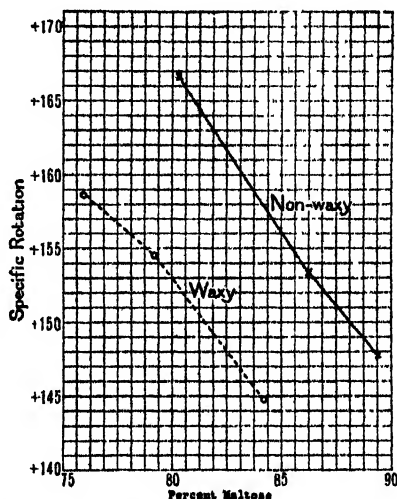


Fig. 2. Specific rotations of the conversion liquids during hydrolysis of the waxy and non-waxy starches with malt amylase. Test 5.

The specific rotation of maltose is  $+138^\circ$ . The values for the dextrans, intermediate products between starch and maltose, are higher. The conversion liquid contains maltose and probably a complex mixture of dextrinous materials. If the optically active substances in the conversion liquids are identical in nature, waxy and non-waxy samples containing the same percentages of maltose will have the same specific rotations. Fig. 2 shows, however, that the values for the common starch stand about  $14^\circ$  higher than those for waxy starch when comparison is made between solutions containing corresponding amounts of maltose. This means that the intermediate compounds between the initial polysaccharide and the sugar are different in the waxy and non-waxy conversion liquids.

These results afford further evidence in support of the conclusion that the waxy and non-waxy starches differ in molecular structure or in the degree of association of their elementary molecules.

*Phosphorus content of waxy starch.*

A number of workers have shown that starch contains phosphorus which is not removed by strong acids and bases at ordinary temperatures or by dialysis. Samec [1914] has concluded that the substance is present in organic combination. Karrer [1925] suggests that the phosphorus compound represents a small impurity in the  $\alpha$ -amylose portion. Weight is lent to this view by the fact that Northrop and Nelson [1916] have isolated from starch an ester containing 5.3 % of phosphorus.

The amount of phosphorus in starch may be accurately and conveniently measured by a modification of the method of Briggs [1922]. The procedure is as follows. 5 g. of starch are incinerated at low heat. The ash is heated with about 80 cc. water on a steam-bath at 100°. 1 cc. of 5 % ammonium molybdate in 5N  $\text{H}_2\text{SO}_4$  and 1 cc. of 1 % quinol slightly acidified with  $\text{H}_2\text{SO}_4$  are added. Identical treatment is given to a blank containing a known amount of a solution of  $\text{KH}_2\text{PO}_4$  containing 1 mg. P per cc. The concentration of phosphorus in the blank is adjusted by preliminary trial to approximate closely that in the unknown. After treating the solutions for 30 minutes, they are cooled and made up to 100 cc. 50 cc. of the blank and the unknown are taken and 1 cc. of a 20 % solution of sodium sulphite is added to each. After standing 10 minutes the solutions are made up to 100 cc. and the tints compared.

Phosphorus determinations carried out in duplicate according to the above procedure gave 0.0192 % for common maize starch and 0.0016 % for the waxy starch. A repetition of the test gave practically the same results, namely, 0.0194 % (common) and 0.0015 % (waxy). Waxy starch, therefore, contains only about one-twelfth as much organic phosphorus as does common maize starch.

The low content of phosphorus in the waxy starch is not due to a deficiency of this element in the endosperm tissue. Using the same methods of analysis as with starch, endosperm meal from common maize was found to have 0.0519 % P while comparable waxy endosperm meal contained 0.0507 %. Duplicate determinations again gave closely similar results.

Practically equal amounts of phosphorus are present in the endosperms of waxy and non-waxy maize but in the former case only a minute amount is combined with the starch.

## SUMMARY.

1. The waxy and non-waxy maize starches contain nearly equal proportions of  $\alpha$ - and  $\beta$ -amylose. It is considered that treatment with barley amylase is a better means of measuring the  $\beta$ -amylose content of a starch than the centrifugation method of Sherman and Baker.

2. When treated with malt amylase the waxy and non-waxy starches both produce maltose. Traces of glucose also occur in the conversion liquids, and the latter are never entirely free from dextrinous materials. The fact that maltose is the main hydrolytic product is taken to mean that the sugar unit entering

into the formation of the two kinds of starches is the same. It is concluded that the waxy gene exerts its differential effect subsequent to the synthesis of the sugar molecules.

3. Under the action of malt amylase common maize starch is hydrolysed to maltose more rapidly than waxy starch. It is suggested that the waxy gene may modify the starch molecule in some way which puts it slightly out of adjustment with malt amylase.

4. It is recognised, however, that the possibility is not definitely excluded that the essential difference between the two starches may be one of degree of association of a common fundamental molecule.

5. During the conversion to maltose with malt amylase waxy starch forms intermediate products of a lower specific rotatory power than does common maize starch. If dissociation and depolymerisation of the starch are completed in the initial stages of the conversion this means that the difference between the waxy and non-waxy carbohydrates lies in the character of their constituent molecules. It may be, however, that some undissociated or polymerised starch persists even until the later stages of digestion. If this is the situation the difference in the optical properties of the respective solutions may be due either to a difference in the constituent units or in the degree of polymerisation or association.

6. Common maize starch of high purity contains over twelve times as much organic phosphorus as does waxy starch prepared in the same way. The low content of phosphorus in waxy starch is not due, however, to a deficiency of this element in the endosperm tissue. Analyses show that the total phosphorus content of waxy and common endosperm meal is nearly the same. The significance of the differences in amount of combined phosphorus is not clear.

A considerable part of the work reported here was done in the Department of the Biochemistry of Fermentation at the University of Birmingham. Grateful acknowledgment is made to Prof. A. R. Ling and Dr D. R. Nanji for instruction in methods and for a critical interest in the problem. The investigation was carried on under the auspices of the National Research Council (U.S.A.), to which the author is indebted for a Fellowship in biology. The author is also indebted to his colleague, Dr K. P. Link, for helpful criticisms in the preparation of the manuscript.

## REFERENCES.

- Armstrong (1924). The carbohydrates and the glucosides. (Longmans, Green and Co., London.)
- Baker and Hulton (1920). *Biochem. J.* **14**, 754.
- Briggs (1922). *J. Biol. Chem.* **53**, 13.
- Brink and Abegg (1926). *J. Genetics*, **11**, 163.
- (1927). *Plant Physiol.* **2**, 101.
- Brown (1904). Laboratory studies for brewing students. (Longmans, Green and Co., London.)
- Karrer (1925). Polymere Kohlenhydrate. (Akademische Verlagsgesellschaft, Leipzig.)
- Ling and Nanji (1923). *J. Chem. Soc.* **123**, 2666.
- Maquenne and Roux (1905). *Compt. Rend. Acad. Sci.* **140**, 1303.
- Meyer (1895). Untersuchungen über die Stärkekörner. (E. Fischer, Jena.)
- Northrop and Nelson (1916). *J. Amer. Chem. Soc.* **38**, 472.
- Pringsheim (1923). Die Polysaccharide. (Julius Springer, Berlin.)
- Samec (1914). *Koll. Chem. Beihefte*, **61**, 23.
- Schryver and Thomas (1923). *Biochem. J.* **17**, 497.
- Sherman and Baker (1916). *J. Amer. Chem. Soc.* **38**, 1885.

# CLXXI. STUDIES ON THE ANTINEURITIC VITAMIN.

## II. THE PROPERTIES OF THE "CURATIVE" SUBSTANCE.

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*(Received September 3rd, 1928.)*

IN preparing extracts of the water-soluble vitamin from rice polishings, we noticed that, unless precautions were taken, alcoholic fermentation readily occurred at laboratory temperature. This fermentation was due to the presence of yeasts, and while yeast has the power of synthesising at least one of the water-soluble vitamins, it has been shown that its growth in wort, or in cultures containing substances which are possibly akin to these vitamins, is superior to that obtained on purely synthetic media [Nelson, Fulmer and Cessna, 1921; Harden and Zilva, 1921].

It was decided, therefore, to ascertain whether fermentation had any deleterious effect upon our extracts.

When our original extract was fractionated by lead acetate, we observed that the filtrate, which contained the curative factor, alone underwent fermentation. The precipitate portion could not be induced to grow yeasts. During the progress of our experiments a paper by Kon and Drummond [1927] appeared, in which they show that the activity of sucroclastic enzymes is not diminished in the polyneuritic pigeon, which, these authors state, is not in accord with the experiments of Farmer and Redenbaugh [1925]. The inference drawn by Kon and Drummond that vitamin B is associated only with protein metabolism, and bears no relationship to the metabolism of fat and carbohydrate, appears at variance with the evidence of Randoin and Simmonet [1924], and of Plimmer, Rosedale and Raymond [1927].

At the time of the arrival of Kon and Drummond's paper, our first series of experiments was giving evidence of deterioration of our extract by fermentation, so that it became of interest to ascertain whether any reason for metabolic disturbance could be obtained from our birds.

Although Plimmer and Rosedale [1922] investigated the distribution of enzymes in the alimentary canal of the normal chicken, we could find no such data for the normal pigeon, so that we were obliged to secure this in order to

compare with pathological cases. Our investigations have been extended to human cases. Our data seem to indicate a difference between normal and beriberi cases as regards the pancreas where we have noticed the absence of trypsin and lipase. Our extract has been similarly examined for enzymes, and while our active (unfermented) preparation invariably shows marked lipoclastic action, we have been unable to establish the presence of proteoclastic enzymes.

#### EXPERIMENTAL.

Our extracts were prepared according to the procedure of Rosedale [1927], by extracting rice polishings in 1 % acetic acid at 40° for 8 hours, filtering, and concentrating *in vacuo* at 40° to such a volume that 1 cc. corresponds to 1 g. of the original polishings. This extract was then treated with lead acetate until no further precipitate could be obtained, when it was filtered. This filtrate was subsequently concentrated to the original volume and constitutes the extract which is the subject of the experiments reported in this paper. The extract can be kept from fermenting for some time in our animal house, which is a cool building, shaded from the sun for the greater part of the day. In the laboratory, where the average temperature is 30°, fermentation usually sets in after standing for 24–36 hours.

*1st series.* On June 22nd four pigeons were started on a white rice diet which was given *ad lib.* and in addition received daily 5 cc. of our extract. On the same day three pigeons suffering from typical polyneuritis were cured by a 5 cc. dose of this batch of extract. This extract was kept in the laboratory and fermentation was evident on the following day. On July 15th it was clear that the appetite of the birds had fallen, and their food was scattered about the cage. On July 25th all the birds showed signs of weakness and only one could mount to the perches. One bird died on July 30th, a second and a third on Aug. 8th, while the fourth was chloroformed on Aug. 19th.

*2nd series.* On Aug. 1st two birds suffering from polyneuritis were cured by 5 cc. each of a fresh batch of extract, 500 cc. of which were set aside in the laboratory and allowed to ferment. On Aug. 8th four pigeons were placed on a diet of white rice. Plimmer, Rosedale and Raymond [1927] have shown that results similar to those obtained in the 1st series may occur when feeding vitamin extracts in too small a proportion in the diet. In view of this and also of the fact that three of the birds in our 1st series had already died, it was decided to give 10 cc. of the fermented extract daily to each bird. The scattering of food was first recorded on Aug. 15th and food consumption was greatly reduced after Aug. 20th. The first death occurred on Sept. 2nd and the succeeding ones on Sept. 4th, 5th and 10th. *Post mortem* examination showed the dropsical condition in the third bird, while the others exhibited the dry condition. The organs of these birds as well as of the last bird in the 1st series were used for the study of enzymes.

These series indicate a loss of vitamin from the fermented extract.

The subsequent series were planned to ascertain whether sterilisation of the extract, which prevented fermentation, would have any effect on the vitamin content. Fresh extracts were first tested in 5 cc. doses upon birds suffering from typical polyneuritis. These proved the extracts to be capable of curing. The extracts were then sterilised either by filtration through a Berkefeld filter or by heating at 95° for 5 minutes. The sterilisation was kindly carried out for us by Prof. W. A. Young in the following way. The filtered extracts were prepared by filtration through previously tested Berkefeld filter candles into heat-sterilised flasks, which were subsequently plugged with sterile cotton wool. The heat-sterilised extracts were sterilised in previously heat-sterilised flasks plugged in the usual way.

Both extracts were distributed into flasks in quantities sufficient for one dose so that a fresh and sterile dose was given on each occasion and any danger of aerial contamination from using a previously opened flask was avoided. Before using, sterility tests were carried out on each batch of extract.

(a) Aerobic cultures. Samples were inoculated into nutrient broth tubes, into glucose broth and litmus milk, and on to agar and Sabouraud's agar slants.

(b) Anaerobic cultures. Samples were inoculated into Robertson's meat medium.

Extracts were used if all test media were sterile at the end of 48 hours, but the tests were kept under observation up to 14 days. No growth was found to occur in any test medium which had not appeared at the end of 48 hours. One batch only was found to be contaminated with aerobic sporing organisms, and was discarded.

*3rd series.* On Sept. 1st eight pigeons were placed upon white rice, four receiving daily 10 cc. of the filtered extract, and the remaining four 10 cc. of the heated extract per bird. All these birds died within 34 days of the commencement of the experiment, and the results are shown in Table I.

Table I.

Date of death	Nature of extract fed	Results of <i>post mortem</i> examination
Sept. 19	Filtered	Dropsy
23	Filtered	Dropsy
24	Heated	Dry, enlarged heart
24	Heated	Dropsy
26	Heated	Dry, enlarged heart, yellow liver
30	Filtered	Dry, enlarged heart, yellow liver
Oct. 2	Filtered	Dropsy
4	Heated	Dropsy

The only case of typical head retraction occurred in the last bird on Oct. 2nd. A dose of 5 cc. of a fresh extract effected a temporary cure of the typical condition, but the bird died on Oct. 4th.

*4th series.* Opportunity was taken of repeating the 3rd series. On June 27th, 1928, eight birds were again placed on a white rice diet. Four each received daily 10 cc. of the filtered extract, and four 10 cc. of the heated extract.

These doses were from batches of extract sterilised by Prof. Young in Aug. 1927, but although they had stood (plugged) at laboratory temperature for many months they showed no visible fermentation or deterioration. The first bird died on July 17th and the last on July 29th. No case of the typical head retraction was observed in this series.

Sterilisation of the extract by filtration and by heat destroys the vitamin.

*Experiments with the enzymes of the alimentary canal.*

The methods employed followed closely those of Plimmer and Rosedale [1922] and are consequently limited to qualitative detection. The various organs were taken from birds as soon after death as possible and several experiments were made with both normal and pathological pigeons. The crop and intestines were cut open and well washed. The mucous membrane was scraped off, ground with sand and extracted with water. The secretion from the proventriculus was placed directly into water and filtered. The pancreas was ground up with sand and extracted with glycerol. After extraction, water was added and the sand filtered off.

As substrates we used for diastase and invertase 10 cc. of a 1 % starch solution and a 1 % sucrose solution respectively. Two portions of each were measured into separate flasks, a known volume of tissue extract being added to one and an equal volume after boiling and cooling to the other to serve as control. After incubation, tests were made for starch with iodine and for glucose in the sucrose series.

*Lipase.* Two equal portions of olive oil were placed in separate flasks and made just alkaline to phenolphthalein. Tissue extract was added to one and an equal volume of boiled extract to the other. The discharge of the colour from the flask containing unboiled extract, in distinction to no change of colour in the boiled, was taken as an indication of the presence of lipase.

*Proteoclastic enzymes* were detected by their action on Congo red fibrin in acid, alkaline and neutral media. Similar volumes of extract and weighed quantities of fibrin were used in the experimental and control tubes. An equal volume of dilute acid or alkali or distilled water was added to each tube. Toluene was employed as preservative. Enzyme action was judged after incubation by the solution of the fibrin. The controls did not show any solution of the fibrin.

Table II.

	Crop		Proventriculus		Pancreas		Intestine	
	Normal pigeon	Poly- neuritic pigeon	Normal pigeon	Poly- neuritic pigeon	Normal pigeon	Poly- neuritic pigeon	Normal pigeon	Poly- neuritic pigeon
Diastase ... ..	+	+slow	0	+slow	++	++	+	+
Invertase ... ..	0	0	0	0	+	+slight	+	+slight
Lactase ... ..	+slight	0	0	0	0	0	0	0
Lipase ... ..	.	.	.	.	+	0	.	.
Proteoclastic in neutral media	0	0	0	0	+slight	0	+slight	0
Proteoclastic in acid media ...	0	0	++	++	+slight	0	0	+slight
Proteoclastic in alkaline media	0	0	0	0	++	0	+	0



The results (Table II) with the normal pigeon agree closely with those of Plimmer and Rosedale for the chicken. The polyneuritic pigeons, which were taken from the series of feeding experiments described above, appear to show differences. In each case sucroclastic enzymes were present. In no case have we been able to observe any tryptic action in the pancreas. In the intestine no proteoclastic enzyme has been detected except in the case of the bird from our 2nd series which died on Sept. 5th. In this case we found a slight hydrolysis of the fibrin in acid medium. The pancreas of this bird also exhibited lipoclastic action, though in no other case were we able to demonstrate the action of lipase in the pancreatic extracts. This bird on *post mortem* examination showed the wet or dropsical condition.

We had the opportunity of carrying out similar experiments with the human pancreas, from both normal and beriberi cases. While trypsin and lipase were readily demonstrated by our methods in the normal cases, neither was observed in the beriberi cases which were reported to us as being of the dry type.

*Enzymes of the extract of rice polishings.*

Owing to the observation of alcoholic fermentation and the presence of yeasts we have made no further experiment with sucroclastic enzymes, but in view of the results with beriberi animals we have attempted to ascertain the presence of proteoclastic enzymes and of lipase. As already stated, we have not been able to show the presence of the former, but lipase has been found to be present. The technique was similar to that employed for tissue extract and we used 20 cc. in each experiment as enzyme solution, each experiment being controlled by an equal volume of boiled extract. In no case did any of our controls show any sign of action.

DISCUSSION.

Our results indicate that during polyneuritis, metabolic disturbances occur involving a certain amount of inactivation of the pancreas. In the "dry" cases we have found failure of both proteoclastic and lipoclastic enzymes, although our methods are unable to show any deterioration of sucroclastic enzymes. Since lipoclastic enzymes appear to be inactive, and as our diet of white rice alone contains but little protein, we cannot conclude with Kon and Drummond that vitamin B bears relationship only to protein metabolism. Plimmer, Rosedale and Raymond [1927] found that the balance between vitamin B and protein was more difficult to demonstrate than that between carbohydrate or fat and vitamin B, but the experiments of Reader and Drummond [1926] leave no doubt that protein is similarly affected.

From our general results we consider that the curative substance has at least some control over the action of pancreatic enzymes. Work in progress by one of us on the bile of polyneuritic animals prevents the conclusion at the moment, however, that the curative substance itself is enzymic.

## SUMMARY.

1. The antineuritic vitamin of an extract of rice polishings is destroyed by fermentation and by sterilisation.
2. The potent curative extract of rice polishings contains sucroclastic and lipoclastic enzymes, but it has not been possible to show the presence of proteoclastic enzymes.
3. In cases of "dry" beriberi, the pancreas has been found incapable of lipoclastic and tryptic digestion.

We should like to take this opportunity of thanking Prof. Young for so kindly carrying out the sterilisation of the extracts for us.

## REFERENCES.

- Farmer and Redenbaugh (1925). *Amer. J. Physiol.* **75**, 27, 45.  
Harden and Zilva (1921). *Biochem. J.* **15**, 438.  
Kon and Drummond (1927). *Biochem. J.* **21**, 632.  
Nelson, Fulmer and Cessna (1921). *J. Biol. Chem.* **46**, 77.  
Plimmer and Rosedale (1922). *Biochem. J.* **16**, 23.  
Plimmer, Rosedale and Raymond (1927). *Biochem. J.* **21**, 913.  
Randoin and Simmonet (1924). *Bull. Soc. Chim. Biol.* **6**, 601.  
Reader and Drummond (1926). *Biochem. J.* **20**, 1256.  
Rosedale (1927). *Biochem. J.* **21**, 1266.

## CLXXII. BACTERIAL OXIDATIONS BY MOLECULAR OXYGEN.

### I. THE AEROBIC OXIDATION OF GLUCOSE AND ITS FERMENTATION PRODUCTS IN ITS RELATION TO THE VIABILITY OF THE ORGANISM.

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*(Received September 15th, 1928.)*

IN recent studies much attention has been directed towards bacterial oxidations in which compounds have been oxidised at the expense of reducible dyes, nitrates, fumarates, etc. It is obvious however that for any organisms growing aerobically the most important hydrogen acceptor is molecular oxygen and that consequently a study of aerobic oxidations is essential if a true picture of the normal life of the cell is to be obtained.

The power to effect the oxidation of their own cell material by molecular oxygen was shown by Callow [1924] to be common to aerobes and facultative anaerobes; this was demonstrated by measuring the oxygen uptake of washed bacteria suspended in buffer solution in a Barcroft manometer; organisms in these conditions take up 5 to 25 cc. of oxygen per g. of dry cell per hour; two organisms only amongst those examined were practically lacking in this faculty, viz. *B. sporogenes*, the only strict anaerobe reported on, and *S. acidilactici*, a facultative anaerobe resembling the strict anaerobes in possessing no catalase.

It is obvious that many aerobes and facultative anaerobes possess the power of oxidising not only the simple carbohydrates but also their fermentation products since aerobic growth is known to occur on lactate, acetate, succinate, glycerol, etc. where these form the only source of carbon [Braun and Cahn-Bronner, 1922; Stephenson and Whetham, 1924]. In the course of the present inquiry we have confined ourselves chiefly to oxidations by *B. coli*, though experiments have also been carried out with *B. alkaligenes* and with *B. sporogenes*; we have sought to discover (1) the degree of oxidation sustained by glucose and some of its fermentation products, viz. lactate, acetate, formate and ethyl alcohol, in conditions highly favourable to aerobic oxidation; (2) the state of the organisms responsible for the process, i.e. whether they be living or dead, and if the former whether multiplying, at rest or dying off.

## METHOD.

The organisms were grown on tryptic broth agar containing 0.5 % sodium lactate; after 24 hours the growth was washed off with phosphate buffer and centrifuged; the organisms were washed twice with Ringer's solution and finally suspended in about 100 cc. of Ringer's solution, aerated for 15 minutes to make an even suspension, and kept at 0°. It is important to use Ringer's solution for this purpose in preference to saline or phosphate buffer as by this means a more actively oxidising suspension is obtained. This stock suspension obtained from 12 agar plates of 5½ inches diameter contains from  $6 \times 10^{10}$  to  $8 \times 10^{10}$  organisms per cc. The suspension is diluted with an equal volume of Ringer's solution before use and 1 cc. employed for each oxidation. The oxidisable materials were usually made up in solutions of  $M/100$  strength; glucose was used in  $M/200$  strength in order to compare directly with  $M/100$  lactate. The purest chemicals obtainable were used; the strength of the acids was checked by titration; in the case of lactic acid a known volume (excess) of  $N/10$  NaOH was added to 10 cc. of approximately  $N/10$  lactic acid and the mixture autoclaved in order to hydrolyse the lactone present and the excess of sodium hydroxide titrated back with HCl and made up so as to contain exactly  $N/100$  sodium lactate; glucose was estimated in  $M/10$  solutions by the method of Wood-Ost.

The oxidations were carried out in the Barcroft differential manometer using the flat-bottomed cups employed in tissue oxidations. The cups were filled as follows.

Left-hand cup 1: 1 cc. buffer (usually  $p_H$  7.4); 1 cc. bacterial suspension; 1 cc. water.

Right-hand cup 2: 1 cc. buffer; 1 cc. bacterial suspension; 1 cc. oxidisable material  $M/100$ . The experiments were carried out in a bath at 40°; 3 minutes were usually allowed for the cups to acquire the temperature of the bath before the taps were closed off; this renders the results somewhat too low, the extent of the error may be gauged by extrapolation. A large number of observations showed that the method was subject to an error of  $\pm 10$  %.

The organisms themselves in the absence of any oxidisable material take up oxygen amounting to about 70 mm.<sup>3</sup> per hour; it is assumed that in our experiments this endogenous uptake is balanced in each pot; i.e. that the presence of the oxidisable material in cup 2 causes no change; no way of checking this assumption has suggested itself but the possibility that our experimental error may be due to an unbalanced endogenous oxidation is not excluded.

*The oxidation of glucose, lactate and pyruvate.*

The amount of oxygen taken up by glucose and lactate is almost identical amounting to 433 mm.<sup>3</sup> of oxygen per cc.  $M/200$  glucose (average of 28 observations) and 438 mm.<sup>3</sup> per cc.  $M/100$  lactate (average of 15 observations) (see Table I).

Table I.

mm. <sup>3</sup> O <sub>2</sub> taken up by 1 cc. <i>M</i> /200 glucose.															
436	426	396	460	456	452	454	380	388	405	510	497	495	476	450	
395	452	412	513	415	431	385	432	396	377	413	412	412.	Average	433	
mm. <sup>3</sup> O <sub>2</sub> taken up by 1 cc. <i>M</i> /100 lactate.															
475	442	400	448	460	490	444	470	463	392	398	460	417	421	405.	
Average 438															

This approximates fairly closely to 4 atoms of oxygen per molecule of lactate or per half molecule of glucose (448 mm.<sup>3</sup>), 6 atoms being required for complete oxidation in each case.

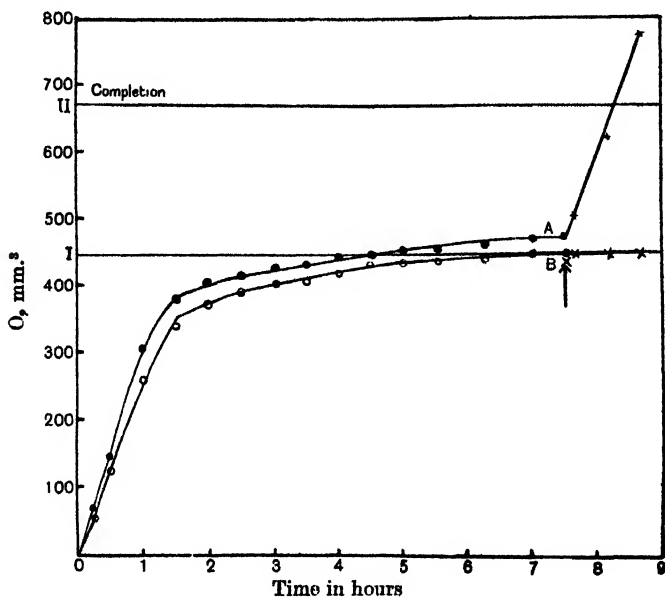


Fig. 1. Oxidation of 1 cc. *M*/200 glucose. At the point indicated by the arrow the curve A shows the effect of the addition of 1 cc. *M*/200 glucose. Curve B shows the effect of adding 1 cc. of fresh bacterial suspension. I and II correspond to the theoretical values for 8 and 12 atoms of oxygen respectively per mol. of glucose.

The initial rate in the case of glucose is somewhat slower than in the case of lactate; this is apparent from the slope of the curve of two oxidations taken simultaneously; it was also demonstrated by oxidising *M*/200 glucose in one Barcroft cup and *M*/100 lactate in the other; the readings of the manometer showed a negative pressure on the lactate side in the early stages of the experiment though later an equilibrium was achieved.

*M*/100 pyruvate (prepared from pyruvic acid freshly distilled at 1 mm. pressure) takes up oxygen approximating to 3 atoms of oxygen per molecule (Fig. 2) agreeing with the fact that lactate passes through the stage of pyruvate during aerobic oxidation by loss of 2 atoms of hydrogen [Stephenson, 1928].

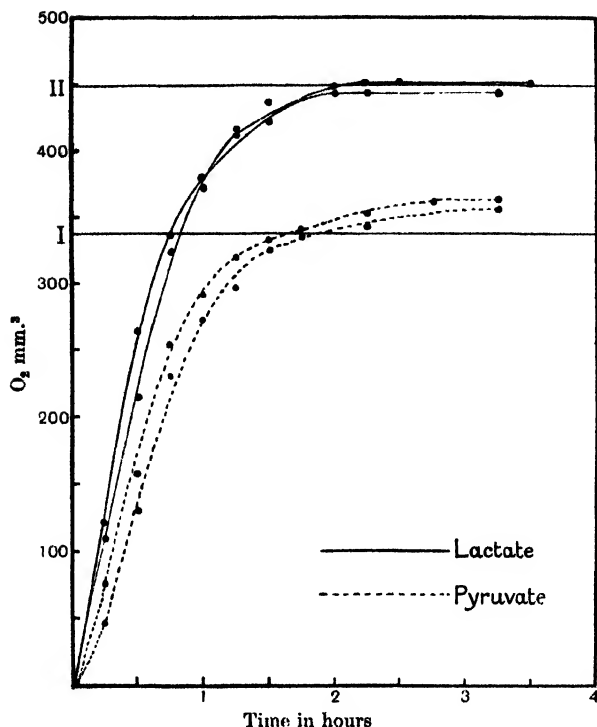


Fig. 2. Oxidation of  $M/100$  lactate and  $M/100$  pyruvate. I and II correspond to the theoretical values for 3 and 4 atoms respectively of oxygen per mol. of pyruvate and lactate.

*The oxidation of acetate, ethyl alcohol and acetaldehyde.*

Acetate is rapidly oxidised, the uptake amounting to 3 atoms of oxygen per molecule of acetate, 4 being required for complete oxidation (Table II, Fig. 3).

Table II.

mm. <sup>3</sup> O <sub>2</sub> taken up by 1 cc. $M/100$ acetate.											
355	386	371	350	308	347	325	350	337	346	314.	Average 335

Ethyl alcohol ( $M/100$ ) remains completely unoxidised; this might be attributed to two causes, either failure of the organism to activate the molecule or to toxic properties; in order to decide between these alternatives  $M/100$  ethyl alcohol was added to  $M/100$  lactate in order to show whether the presence of the former at this concentration inhibited the oxidation of the latter; no inhibition was observable (see Fig. 4) and we must therefore conclude that its failure to become oxidised is due to the lack of the appropriate enzymes in the organism concerned.

Acetaldehyde also gives no oxygen uptake; this is not due to its toxic nature since in  $M/100$  and  $M/200$  concentrations it exerts no inhibition on

the oxidation of  $M/200$  glucose (Fig. 5). Its failure to become oxidised is unexpected in view of the generally accepted theory that ethyl alcohol and acetic acid arise in fermentation through the dismutation of acetaldehyde; this would lead us to expect that in the conditions of our experiments 2 molecules of acetaldehyde would give rise to one molecule of ethyl alcohol which would remain unoxidised and one molecule of acetic acid which would take up 3 atoms of oxygen as already shown.

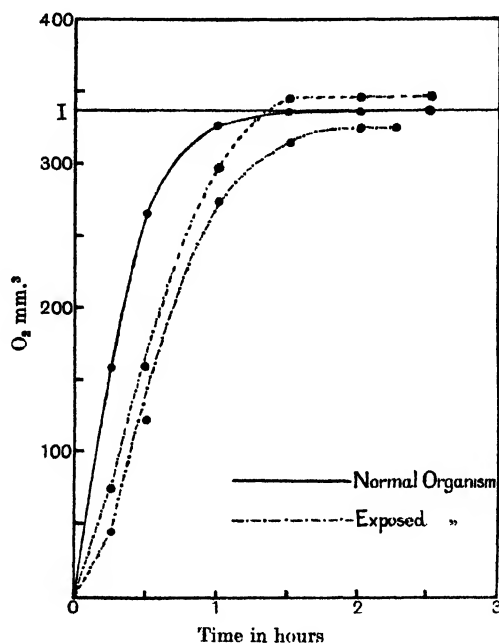


Fig. 3. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc.  $M/100$  acetate.

Time of exposure (in mins.)	...	0	20
Total number of organisms present		$3.04 \times 10^{10}$	$3.04 \times 10^{10}$
Number of viable organisms present		$1.04 \times 10^{10}$	$2.80 \times 10^7$

I corresponds to the theoretical value of 3 atoms of oxygen per mol. of acetate. Four atoms represent complete combustion.

The fate of the acetaldehyde under the influence of the organism was then investigated. In order to avoid the possibility of loss due to evaporation these experiments were carried out in 25 cc. Erlenmeyer flasks, the same quantities of reagents being taken as in the Barcroft experiments; these were cooled to  $0^\circ$  before being mixed, and immediately corked and sealed with paraffin wax; they were then incubated at  $37^\circ$  for 6 hours. The acetaldehyde was estimated by the method of Friedemann, Cotonio and Shaffer [1927] in control experiments without incubation both in the presence and absence of the organism; as the presence of the bacterial suspension made no appreciable difference to the results it was not considered necessary to distil off the acetaldehyde before making the estimation.

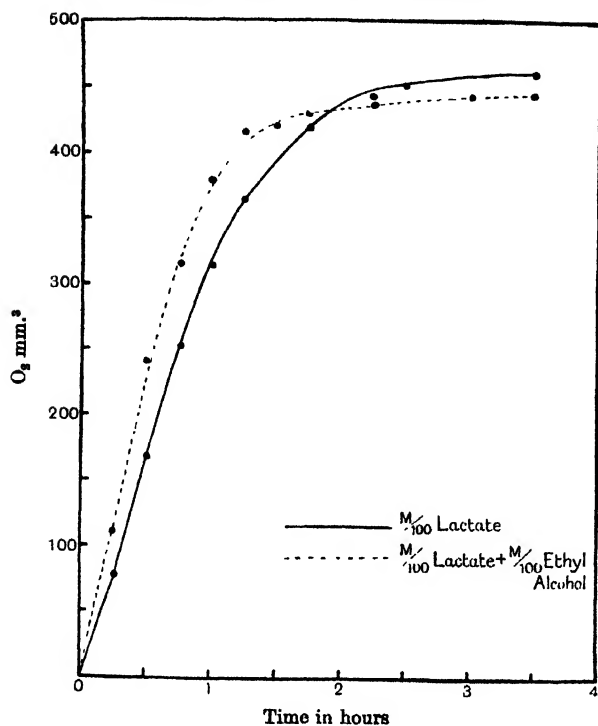


Fig. 4. Effect of alcohol on oxidation of  $M/100$  lactate.

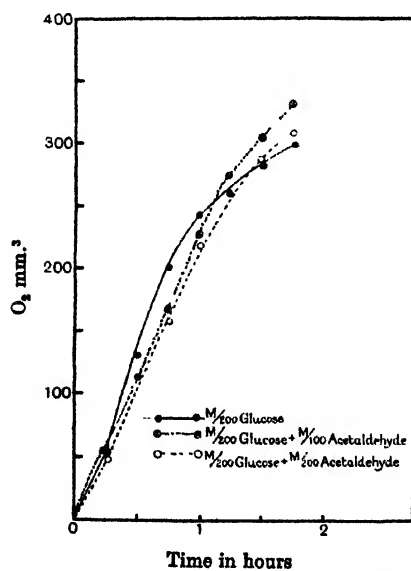


Fig. 5. Effect of acetaldehyde on the oxidation of  $M/200$  glucose.



Table III.

	cc. $N/1000$ iodine	$A \times 0.05 =$ cc. $M/100$ acetaldehyde
1 cc. $M/100$ acetaldehyde + 1 cc. Ringer + 1 cc. water	18.1 } 18.0 }	18.05
1 cc. $M/100$ acetaldehyde + 1 cc. Ringer + 1 cc. bacterial suspension	18.5 } 18.5 }	18.5
1 cc. $M/100$ acetaldehyde + 1 cc. Ringer + 1 cc. boiled bacterial suspension	18.5	0.925
Error due to bacterial suspension	0.45	0.022
1 cc. $M/100$ acetaldehyde + 1 cc. Ringer + 1 cc. boiled bacterial suspension incubated in stoppered flask for 6 hours	18.8 } 18.7 }	18.75
1 cc. $M/100$ acetaldehyde + 1 cc. Ringer + 1 cc. bacterial suspension incubated in stoppered flask for 6 hours	10.2 } 10.1 }	10.15
Disappearance of acetaldehyde due to incubation with bacterial suspension = 47 %	—	0.937 - 0.507 = 0.43

From the figures in Table III it is clear that at the temperature and concentration of our experiments acetaldehyde undergoes no diminution in the absence of the bacteria nor in the presence of boiled bacteria; in the presence of active organisms about 47 % disappears in the course of 6 hours; in the absence of an oxygen uptake this cannot be due to an oxidation unless this be balanced by a simultaneous reduction which must give an oxidisable material (acetic acid), whether it be due to polymerisation or to some other cause is undecided.

#### *The oxidation of formate.*

Formate alone among the substances studied is oxidised to completion (Fig. 6). This is noteworthy in view of the fact that Pakes and Jollyman [1901] found that *B. coli*, in the semi-aerobic conditions obtaining in a test-tube and in the presence of peptone, decomposes formates with liberation of hydrogen; had this occurred in our experiments we should have obtained an apparently lower oxygen uptake than that required for complete oxidation due to the positive pressure exerted by the liberated hydrogen. In order to

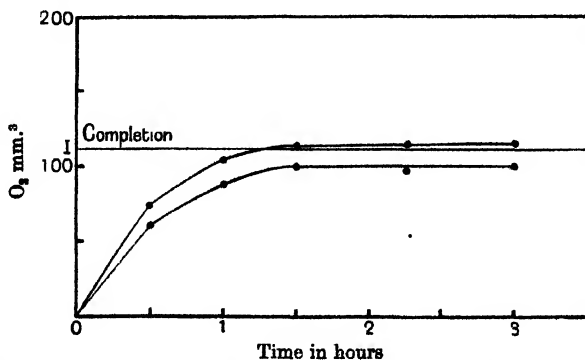


Fig. 6. Oxidation of 1 cc.  $M/100$  formate. 1 corresponds to the theoretical value for 1 atom of oxygen per mol. formate, i.e. complete oxidation.

be sure that this did not occur we repeated the oxidations at an acid reaction ( $p_H$  5.75) still obtaining an oxygen uptake corresponding to complete combustion. We also carried out three experiments in an atmosphere of nitrogen; in these anaerobic conditions liberation of hydrogen would be unbalanced by absorption of oxygen and therefore be clearly apparent; for this purpose we used a Barcroft apparatus having a side-tube and tap inserted above the junction of the cup and the manometer; by means of this tube the cups were evacuated after the solutions had been placed in them and nitrogen was passed in through a 3-way tap; this was repeated three times, the taps were then closed and the apparatus was placed in the water-bath; after 5 minutes the taps were opened for a moment for equilibration and the experiment was carried on as usual; no evolution of hydrogen was observed in any of the three experiments and we therefore concluded that in the conditions of our oxidations no evolution of free hydrogen occurred. It is noteworthy that the oxidation of formate was not more rapid than that of other substrates studied; this is interesting in view of the fact that in the case of methylene blue the rate of reduction by formate is 700 times that of acetate though only 1.2 times that of lactate [Quastel and Whetham, 1925].

*The oxidation of glycollate and oxalate.*

Glycollate is very slowly oxidised, the oxidation ceasing when 2 to 3 atoms of oxygen have been taken up per molecule of glycollate, 3 being required for complete oxidation. Oxalate is unoxidised; this again is not due to toxicity since  $M/100$  oxalate causes no inhibition in the oxidation of  $M/200$  glucose. Malonic acid is also unoxidised.

All experiments with boiled bacterial suspension were negative.

*Experiments with B. alkaligenes and B. sporogenes.*

*B. alkaligenes* was selected as being an example of a strict aerobe and therefore more likely than *B. coli* to carry oxidations to completion. This organism differs from *B. coli* in being unable to oxidise glucose; this accords with the fact that it is unable to activate glucose to act as a hydrogen donor in the presence of methylene blue [Quastel and Wooldridge, 1925]. Its behaviour with lactate however corresponds with that of *B. coli*, i.e. 1 molecule of lactate takes up approximately 4 atoms of oxygen, 6 being required for complete oxidation. With acetate and formate also its behaviour corresponds with that of *B. coli*, i.e. three-fourths oxidation of acetate and complete oxidation of formate.

*B. sporogenes* was chosen as an example of a strict anaerobe. The organism was grown in tryptic broth to which 0.1 % of cysteine hydrochloride previously neutralised was added. The organism was grown for 48 hours and centrifuged and washed in the usual way; the suspension consisted mainly of vegetative cells with but few spores.

No oxygen uptake on any of the substrates tested was obtained. The following substances were tried:

broth,  $M/200$  glucose,  $M/100$  lactate, pyruvate and formate.

In view of the probability that aerobic oxidations involve the activation both of the hydrogen donator and of molecular oxygen we considered that the failure to obtain oxidations by *B. sporogenes* might be due to its inability to effect the activation of oxygen; if this were the case the oxidation might be brought about by the addition of an autoxidisable substance capable of re-oxidation by molecular oxygen as is the case with the lactic dehydrogenase and methylene blue [Stephenson, 1928]. Accordingly we tried the addition of  $M/3000$  methylene blue to lactate, pyruvate and also  $M/3000$  glutathione (reduced form) to glucose and broth. No significant oxygen uptake was observed in any of these cases.

It has been suggested [M'Leod and Gordon, 1923] that strict anaerobes produce hydrogen peroxide through the aerobic oxidation of their substrates and that this accumulates owing to the absence of catalase from these organisms. If this were so it must surely be evidenced by an oxygen uptake whether the peroxide accumulates as in the case of the *Pneumococcus* or whether it causes secondary oxidations as in the case of the xanthine oxidase system [ThurLOW, 1925]. An oxygen uptake equivalent to a production of hydrogen peroxide amounting to 1 part in 50,000 would be clearly demonstrated in our experiments; if peroxide production occurs therefore, it must be within these limits.

We therefore conclude that in the case of this strict anaerobe the oxidative mechanisms are either absent or require totally different conditions for their manifestation than in the case of aerobes and facultative anaerobes.

#### *The influence of hydrogen ion concentration.*

The influence of hydrogen ion concentration on the rate of oxidation of lactate by *B. coli* was studied, the rate being measured by the oxygen uptake during the first half hour of the experiment. The exchange of phosphate for phthalate and borate buffers of the same  $p_H$  made no difference to the rate as is shown by the following example.

#### *Experiment.*

Time (hrs.)	$p_H$	Buffer	O <sub>2</sub> uptake 1 cc. $M/100$ lactate (mm. <sup>3</sup> )
3	5.75	Phosphate	391
3	5.88	Phthalate	399
3	7.95	Phosphate	355
3	7.90	Borate	359

The  $p_H$  of the buffers was determined electrometrically. It is seen (Fig. 7) that an optimum occurs about  $p_H$  5.6 but that little fall occurs on passing to the alkaline side till  $p_H$  9 is reached; below  $p_H$  4.2 and above  $p_H$  11.0 no oxidation occurs; a similar curve for glucose follows approximately the same course. This corresponds with the findings of Sherman and Holm [1922], who

showed that in optimum salt concentration the growth rate varied but little between  $p_H$  5.3 and 8.3.

*The physiological condition of the organisms (B. coli) responsible for the oxidation.*

As is apparent from the curves, the course of the oxidations exhibits in the main the features of an enzyme system, there being no evidence that the reaction is dependent on bacterial multiplication; it remains however to determine whether the cells responsible for the oxidation are alive or dead in the bacteriological sense, *i.e.* capable or incapable of multiplication when subcultivated; and if the former, whether during the oxidation period they are static, multiplying or dying off.

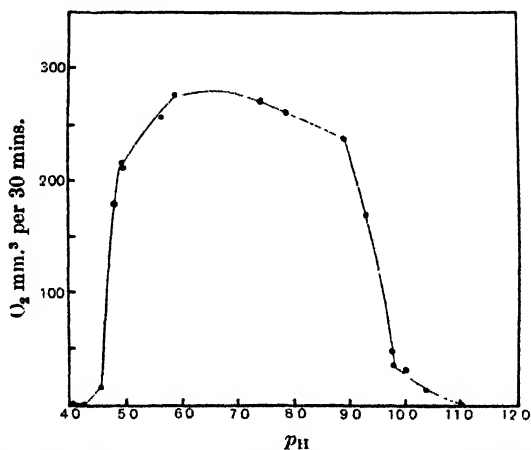


Fig. 7. Effect of hydrogen ion concentration on the rate of oxidation of lactate.

To determine these points both total and viable counts were made on the suspension and viable counts were repeated after the organism had been shaken in the Barcroft apparatus (1) with and (2) without oxidisable substrate, *i.e.* in each Barcroft cup at the end of the experiment. The total counts were carried out by a method described by Wilson [1922] in which the suspension, after suitable dilution in 1 % phenolised saline, is counted directly in a specially calibrated slide using dark ground illumination. The viable count was done by the roll tube method, serial dilutions of the suspension being made in sterile Ringer's solution and drops being withdrawn by means of standard dropping pipettes into test-tubes containing about 2 cc. of nutrient agar at 45° which was immediately rolled.

#### *Example of results.*

##### *Total count.*

- |           |   |          |                   |
|-----------|---|----------|-------------------|
| 1 cc.     | original suspension + 9 cc. phenolised saline | = 10 cc. | Tube (1)          |
| 1 cc. (1) | „ + 9 cc.                                     | „        | = 10 cc. Tube (2) |
| 1 cc. (2) | „ + 9 cc.                                     | „        | = 10 cc. Tube (3) |

The organisms in tube (3) were counted direct on the slide, 100 small squares being counted in each case and the average number of organisms in one small

square thus obtained. The results of three counts were as follows: 4.30, 3.86, 4.12; average 4.09.

The volume of one small square being  $1/20,000$  mm.<sup>3</sup> the number of organisms per 0.5 cc. of the original suspension (which is the quantity used in each Barcroft cup) =  $4.09 \times 10^{10}$ . (Error  $\pm 5$  %.)

#### *Viable count.*

1 cc.	suspension from Barcroft cup	+ 9 cc. Ringer	= 10 cc. Tube (1)
1 cc. (1)	"	+ 9 cc. "	= 10 cc. Tube (2)
1 cc. (2)	"	+ 9 cc. "	= 10 cc. Tube (3)
1 cc. (3)	"	+ 9 cc. "	= 10 cc. Tube (4)
1 cc. (4)	"	+ 9 cc. "	= 10 cc. Tube (5)
1 cc. (5)	"	+ 9 cc. "	= 10 cc. Tube (6)

Serial no. of tube	Drops taken	Colonies found	Colonies per drop	Colonies per drop $\times 76.1^* =$ colonies per cc.
6	20	270	13.5	1027
6	20	268	13.4	
6	10	137	13.7	

\* 76.1 = drops of Ringer's solution per cc. delivered by dropping pipette.

In considering the results of our viable count we have allowed for a possible error of  $\pm 10$  %; differences of less than 20 % have therefore no significance.

It is noteworthy that contaminating colonies were only rarely observed in any of the roll tubes.

We first determined whether keeping the organisms aerated in a Barcroft apparatus at 40° resulted in any change in the number of living cells. 1 cc. bacterial suspension in Ringer's solution, 1 cc. phosphate buffer ( $p_H$  7.4) and 1 cc. water were placed in each cup of the apparatus and shaken for 4 hours; 1 cc. was then withdrawn from each cup, diluted, and a viable count taken and compared with the count before treatment; the results given throughout this paper represent the number of organisms in the Barcroft cup, see Table IV.

Table IV.

Number of organisms in each cup of Barcroft apparatus				
Total	Viable			Increase
	Before experiment	After shaking in buffer for 4 hours with no oxidisable material		
$4.30 \times 10^{10}$	$3.09 \times 10^9$	$3.16 \times 10^9$	$4.92 \times 10^9$	49 %
$4.12 \times 10^{10}$			$4.72 \times 10^9$	
$3.86 \times 10^{10}$	$3.24 \times 10^9$		$4.53 \times 10^9$	

From Table IV it is clear that mere aeration in Ringer and buffer at 40° causes some viable organisms to divide, the results agreeing with the supposition that about half the number of viable organisms divide once in 4 hours—the course of an average experiment. We next ascertained whether the presence of oxidisable material causes a further increase in this multiplication.

For this purpose counts were made at the end of the oxidation experiments in each cup, the increase on the left representing that which took place in Ringer's solution and buffer, that on the right in Ringer's solution, buffer and oxidisable material. The results are given in Table V.

Table V. *Number of organisms in each cup of Barcroft apparatus.*

Oxidisable material	Total	Beginning of experiment		End of experiment		% increase during experiment	
		Viable		Viable		Viable	
		Left cup without oxidisable material	Right cup with oxidisable material	Left cup without oxidisable material	Right cup with oxidisable material	Left cup without oxidisable material	Right cup with oxidisable material
Glucose <i>M</i> /200 3½ hrs.	4.09 × 10 <sup>10</sup>	3.16 × 10 <sup>9</sup>	3.16 × 10 <sup>9</sup>	5.58 × 10 <sup>9</sup>	7.35 × 10 <sup>9</sup>	76	132
Glucose <i>M</i> /200 7 hrs.	"	"	"	4.74 × 10 <sup>9</sup>	7.35 × 10 <sup>9</sup>	50	132
Glucose <i>M</i> /200 5 hrs.	3.04 × 10 <sup>10</sup>	1.04 × 10 <sup>10</sup>	1.04 × 10 <sup>10</sup>	1.70 × 10 <sup>10</sup>	2.06 × 10 <sup>10</sup>	63	98
Lactate <i>M</i> /100 2½ hrs.	4.09 × 10 <sup>10</sup>	3.16 × 10 <sup>9</sup>	3.16 × 10 <sup>9</sup>	4.29 × 10 <sup>9</sup>	5.46 × 10 <sup>9</sup>	41	73
Lactate <i>M</i> /100 2½ hrs.	"	"	"	5.25 × 10 <sup>9</sup>	6.06 × 10 <sup>9</sup>	66	92
Formate <i>M</i> /100 6½ hrs.	"	"	"	4.95 × 10 <sup>9</sup>	4.92 × 10 <sup>9</sup>	57	59

From Table V it is clear that aeration at 40° without oxidisable material causes an increase of 40-76 %; the presence of oxidisable material raising this to 59-132 %. One may regard these increases as being due to a proportion of the viable organisms being in a state almost ready to divide and requiring only the stimulus of the temperature and salts to cause them to complete one division; a further number require the extra stimulus of the carbon food material. It must be pointed out that no nitrogenous food material is present except that obtained from the dead organisms.

The next question to be answered is whether the oxygen uptake measured is due to the living or to the dead cells; in order to ascertain this we reduced the number of living organisms by exposure to ultra-violet light from a mercury lamp. The lamp used was a K.B.B. atmospheric quartz mercury vapour lamp; the culture in Ringer's solution was placed in a quartz test-tube enclosed in a second quartz tube to avoid overheating and clamped 6 inches from the lamp; exposures of 20 min. and 30 min. were made, the suspension being turned and shaken at intervals; after exposure the culture was diluted and a viable count made; oxidations were carried out with the suspension immediately after exposure and the rates compared with those of the normal organism. Tables VI and VII show the effect of the exposure on the number of live organisms; 20 minutes' exposure leaves approximately 2.74 organisms alive out of 1000; 30 minutes' exposure leaves 2 in 10,000. Fig. 8 shows the rate of oxygen uptake on glucose, by untreated organisms and by those

exposed for 20 and 30 minutes respectively. The greatest effect is observed in the initial stages, the uptake at the end of 30 minutes being reduced in the case of the suspension previously exposed for 20 minutes to slightly over one-half and in that exposed for 30 minutes to slightly under one-half; by the end of one hour the rates are approximately equal. Since the number of living organisms has been divided by 370 and 5000 respectively by the 20 minutes' and 30 minutes' exposure it is quite clear that the living organisms exercise no

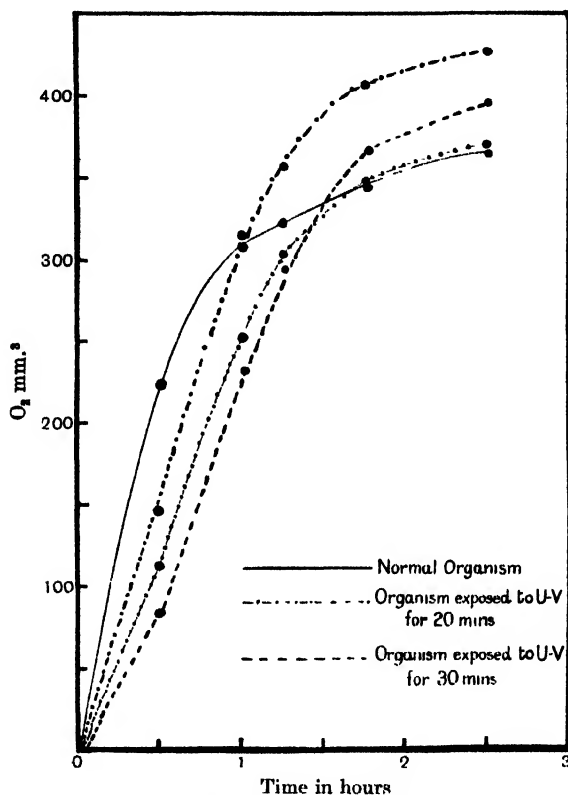


Fig. 8. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. *M*/200 glucose.

Time of exposure (mins.)	0	20	20	30
Total number of organisms present	$3.04 \times 10^{10}$	$3.04 \times 10^{10}$	$3.04 \times 10^{10}$	$3.04 \times 10^{10}$
Number of viable organisms present	$1.04 \times 10^{10}$	$2.85 \times 10^7$	$2.80 \times 10^7$	$2.16 \times 10^6$

measurable influence on the oxidation process, the very slight falling off in the rate by the exposed cultures being attributable to a slight injury to both dead and living organisms similar to that experienced by any enzyme system on exposure to ultra-violet light. This experiment also shows that the increase in viable organisms in the Barcroft apparatus during the process of oxidation has no measurable connection with the oxygen uptake, since in the case of organisms previously exposed to ultra-violet light the oxidation is attended by a substantial death-rate in the few surviving organisms (Tables VI and VII).

The oxidation of lactate by exposed suspensions suffers much the same slight diminution as in the case of glucose (Fig. 9); that of formate is reduced to about one-half (Fig. 10) but even in this case shows no quantitative relationship to the number of living cells. It is interesting to compare the effect of reducing the number of living organisms by radiation with that of reducing the total number by dilution (Fig. 11). In the latter case the rate of oxidation is roughly proportional to the number of organisms present.

Table VI.

Time of exposure (min.)	Number of viable organisms in Barcroft cup before and after exposure to ultra-violet light		
	Before shaking	After shaking	
		Without oxidisable material	With oxidisable material
0	$1.04 \times 10^{10}$	$1.70 \times 10^{10}$	$2.06 \times 10^{10}$
20	$2.85 \times 10^7$	$6.61 \times 10^6$	$3.57 \times 10^6$
30	$2.16 \times 10^6$	$5.35 \times 10^6$	$1.84 \times 10^6$

Table VII.

Time of exposure (mins.)	Relative number of viable organisms before and after exposure to ultra-violet light			
	Before exposure	After exposure		
		Before shaking in Barcroft apparatus	After shaking in Barcroft apparatus	
			Without oxidisable material	With oxidisable material
20	1000	2.74	0.63	0.51
30	1000	0.207	0.034	0.017

We may conclude from these experiments that the oxidation phenomena studied are the work of dead equally with living organisms.

*Problem of the incomplete oxidation of glucose, lactate and acetate.*

These experiments show that *B. coli* is capable of oxidising glucose and the fermentation products which it produces, with the exception of ethyl alcohol and acetaldehyde. The problem opened up by our results is contained in the observation that neither glucose, lactate nor acetate is oxidised to completion, the two former taking up about two-thirds and the latter about three-fourths the amount of oxygen required for complete oxidation. In the case of glucose and lactate this ratio has been found to persist throughout very varying conditions; it holds throughout the range of hydrogen ion concentration at which oxidation occurs and also at varying dilutions; it is unaffected by growing the organisms anaerobically instead of on agar plates or by killing off the living organisms by ultra-violet light.



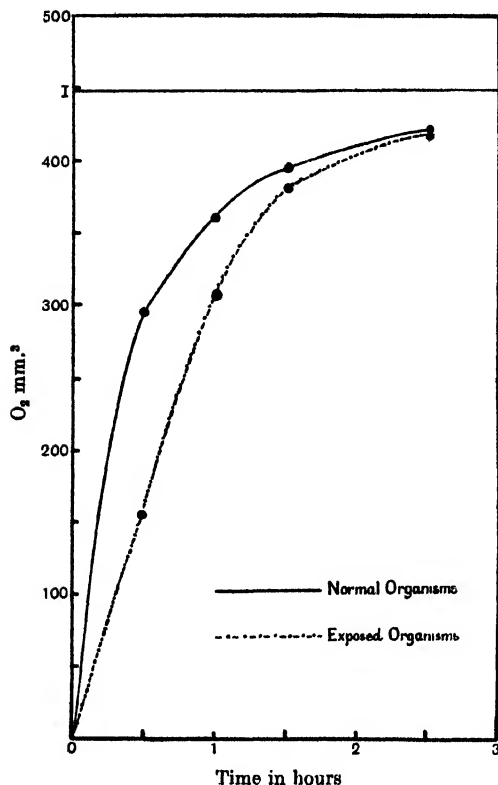


Fig. 9. Effect of the exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. *M*/100 lactate.

Time of exposure (min.)	...	...	0	20
Total number of organisms present			$3.04 \times 10^{10}$	$3.04 \times 10^{10}$
Number of viable organisms present			$1.04 \times 10^{10}$	$2.80 \times 10^7$

I corresponds to the theoretical value for 4 atoms of oxygen per mol. of lactate; 6 atoms represents complete combustion.

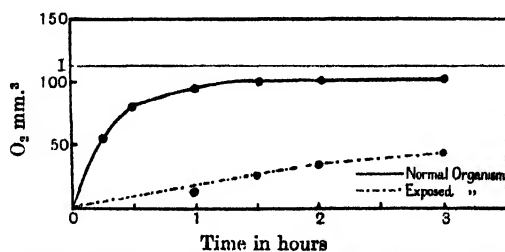


Fig. 10. Effect of exposure of the bacterial suspension to ultra-violet light on the subsequent oxidation of 1 cc. *M*/100 formate.

Time of exposure (min.)	...	...	0	20
Total number of organisms present			$3.04 \times 10^{10}$	$3.04 \times 10^{10}$
Number of viable organisms present			$1.04 \times 10^{10}$	$2.80 \times 10^7$

I represents the theoretical oxygen uptake for complete oxidation.

Several explanations for this behaviour suggested themselves. The first was the possibility that the oxidation fell short of completion owing to the destruction of the enzymes responsible. This was tested by removing the Barcroft cups after the oxidation had ceased and adding to each 1 cc. of fresh bacterial suspension; no further oxidation was however obtained (Fig. 1, p. 1370). The alternative device of adding 1 cc. of fresh oxidisable material to cup 2 and 1 cc. of water to cup 1 resulted in an immediate renewed uptake of oxygen at approximately the original rate (Fig. 1). The idea that the oxidation had ceased owing to the destruction of the enzyme was therefore untenable.

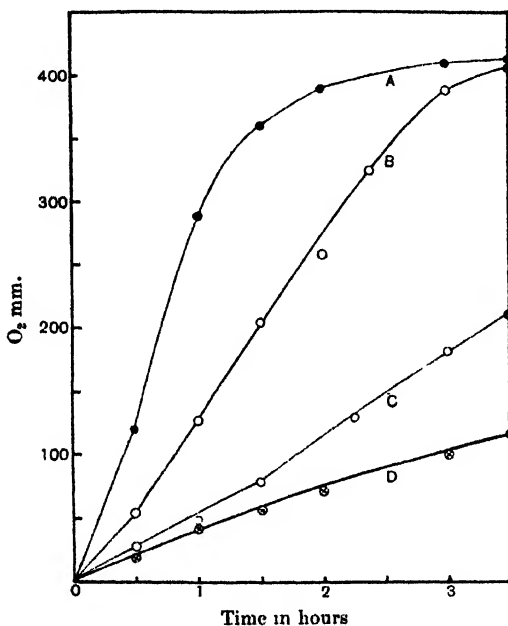
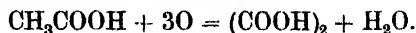


Fig. 11. Effect of the dilution of the bacterial suspension on the oxidation of 1 cc. of *M*/200 glucose.

	Total number of organisms $\times 10^{10}$
A = Normal dilution	3.04
B = $A/2$	1.52
C = $A/5$	0.608
D = $A/10$	0.304

A second explanation which suggested itself was that by the time two-thirds of the glucose or lactate was oxidised the concentration had fallen below the threshold at which oxidation could occur. If this were the case, oxidation of *M*/600 (or less) glucose should not occur; oxidation however occurs with *M*/800 glucose, two-thirds the theoretical complete uptake of oxygen being again achieved (Fig. 12). It seems fairly certain therefore that oxidation does not cease whilst a proportion of the original substrate remains unoxidised and we must look in another direction for an explanation of the constant incomplete uptake.

In the case of acetic acid the uptake corresponds very closely to that required to oxidise acetic acid to oxalic acid:



Moreover, since oxalate itself is unoxidised by the organism it seemed likely that it might be accumulating as an end-product; should this be the case it would be present in the Barcroft cup at the end of the oxidation in *M*/300 strength. The possibility of detecting oxalate in this concentration was explored; for this purpose 1 cc. buffer + 1 cc. water + 1 cc. *M*/100 oxalate were acidified with one drop of concentrated sulphuric acid and titrated with *N*/100  $\text{KMnO}_4$  using a micro-burette; the presence of 1 cc. of bacterial suspension strongly reduces permanganate and it was therefore necessary to remove the organisms before titration; to do this the contents of the Barcroft cup were transferred by means of a curved pipette to a small centrifuge cup, the Barcroft cup was washed out twice with 1 cc. of water, and 1 cc. of 20 % phosphotungstic acid added to the combined contents and washings and the whole centrifuged until the supernatant liquid was clear; 5 cc. of the liquid was then withdrawn, heated to boiling and titrated with *N*/100  $\text{KMnO}_4$ .

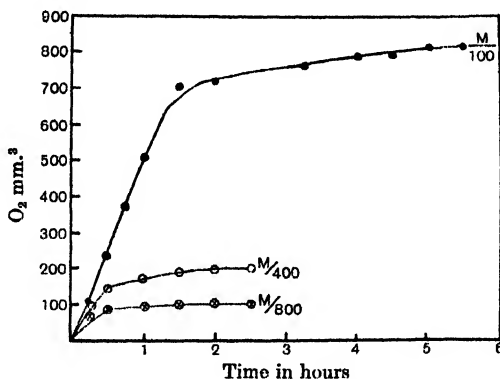


Fig. 12. Oxidation of 1 cc. of *M*/100, *M*/400 and *M*/800 glucose.

### Results.

		cc. <i>N</i> /100 $\text{KMnO}_4$	
1 cc. <i>M</i> /100 oxalate } 1 cc. buffer } 1 cc. water }		1.75	1.70
		1.70	
		1.65	
		1.75	
		1.65	
1 cc. <i>M</i> /100 oxalate } 1 cc. buffer } 1 cc. bacterial suspension } 2 cc. water }	The whole centrifuged and 5 cc. of the super- natant liquid taken for titration	1.47	$1.50 \times \frac{2}{3} = 1.80$
		1.58	
		1.54	
		1.50	
		1.42	

The experiment was then repeated with the contents of Barcroft cups in which 1 cc. *M*/100 and 1 cc. *M*/50 acetate had been oxidised; no measurable reduction of the permanganate occurred and we have therefore provisionally abandoned the hypothesis that acetate is oxidised to oxalate.

As previous work has shown that in the case of the Timothy Grass bacillus the addition of acetate to the media greatly increased the fat content of the cell [Stephenson and Whetham, 1922] it seemed worth while to test whether in our experiments the unoxidised acetate might be giving rise to fat. For this purpose the oxidation was carried out in a 1000 cc. Erlenmeyer flask through which a rapid stream of air was led; two experiments were made, one with and one without acetate; the contents of the flasks were as follows:

Flask 1	Flask 2, control, half-quantity
100 cc. bacterial suspension	50 cc. bacterial suspension
100 cc. Ringer's solution	50 cc. Ringer's solution
200 cc. phosphate buffer $p_H$ 7.4	100 cc. buffer
200 cc. acetate (= 1.2 g. acetic acid)	100 cc. water

As the experiment was run for 20 hours special precautions were taken to ensure that no contaminating organisms were present; the bacteria were grown on agar in Roux bottles, washed off with sterile Ringer's solution and centrifuged and washed with sterile precautions; the suspension was plated at the end of the experiment and found to be in pure culture. The experimental flasks and the rubber stoppers fitted with entrance and exit tubes were separately sterilised and the sterile solutions together with the bacterial suspension introduced into the flasks by means of sterile pipettes; finally the stoppers were placed in position and sealed with paraffin wax. The entrance and exit tubes were protected from contamination by cotton wool plugs and the exit tube was fitted with a Kjeldahl trap to act as a condenser and prevent the plug from becoming wet. The liquid in the flasks was aerated by a steady stream of air passed through by means of an aspirator. The experiment was run for 20 hours; a drop was then withdrawn, diluted and plated and the culture was found to be pure. The whole was then centrifuged and the bacterial deposit dried *in vacuo* at 96° treated with 98 % alcohol, evaporated to dryness and then extracted with ether in a Soxhlet apparatus. The supernatant liquid from the centrifuge cups was passed through a bacterial filter, evaporated on a water-bath to about 50 cc. and extracted from neutral solution with ether in a liquid extractor.

### Results.

	Experiment (with acetate) (mg.)	Control without acetate (mg. $\times$ 2)
Fat from bacteria	32.5	50
Fat from solution	38.0	64

From these results there is clearly no indication that the unoxidised acetate gives rise to fat. It is now clear that the fate of the unoxidised portions of glucose, lactic and acetic acids can only be investigated by means of work on a large scale and we have therefore decided to allow it to form the subject of a separate investigation.

## SUMMARY.

1. *B. coli* is capable of oxidising, by means of molecular oxygen, glucose and certain of its products of fermentation, viz. lactate, pyruvate, acetate, and formate; ethyl alcohol and acetaldehyde are not oxidised, though in the concentrations used they do not act as inhibitors to the oxidation of other substances.

2. The oxidation of formate is carried to completion; that of glucose and lactate to two-thirds and of acetate to three-fourths of completion.

3. *B. alkaligenes* differs from *B. coli* in being unable to oxidise glucose; its behaviour to lactate, acetate and formate is similar to that of *B. coli*. *B. sporogenes* is unable to oxidise any of the substrates tried.

4. Oxidations by *B. coli* are not dependent on living cells; these may be originally present in proportions varying from one-third to one-tenth of the total number of organisms; on reducing their number by exposure to ultra-violet radiation to 0.27 % and 0.021 % of their original number the rate of oxidation is only slightly affected.

We are particularly indebted to Dr G. S. Wilson, of the London School of Hygiene, for personal instruction in the art of counting bacteria. We should also like to record our thanks to Sir F. G. Hopkins for his interest in our work. One of us (M. S.) is indebted to the Medical Research Council for a full-time grant.

## REFERENCES.

- Braun and Cahn-Bronner (1922). *Biochem. Z.* **131**, 226.  
Callow (1924). *Biochem. J.* **18**, 507.  
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 355.  
M'Leod and Gordon (1923). *J. Path. Bact.* **26**, 332.  
Pakes and Jollyman (1901). *J. Chem. Soc.* **79**, 386.  
Quastel and Whetham (1925). *Biochem. J.* **19**, 520.  
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.  
Sherman and Holm (1922). *J. Bact.* **7**, 465.  
Stephenson (1928). *Biochem. J.* **22**, 606.  
— — and Whetham (1922). *Proc. Roy. Soc. Lond. B*, **93**, 262.  
— — and Whetham (1924). *Biochem. J.* **18**, 498.  
Thurlow (1925). *Biochem. J.* **19**, 176.  
Wilson (1922). *J. Bact.* **7**, 405.

# CLXXIII. OBSERVATIONS ON THE FUNCTION OF PEROXIDASE SYSTEMS AND THE CHEMISTRY OF THE ADRENAL CORTEX.

## DESCRIPTION OF A NEW CARBOHYDRATE DERIVATIVE.

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*(Received October 8th, 1928.)*

### INTRODUCTION.

EIGHT years ago observations were made on the adrenalectomised animal which gave the impression that the adrenal cortex is in some way involved in the mechanism of biological oxidation. A detailed study of the biological oxidation was begun in the hope that this study might lead to the understanding of the function of the interrenal system.

Through several years different animal, vegetable and synthetic oxidising systems were studied [Szent-Györgyi, 1924, 1, 2; 1925, 1, 2; 1927, 1, 2], but no connection could be found between these and the function of the adrenal cortex.

Finally the peroxidase system was examined. Evidence is presented in the present paper that the adrenal cortex is connected in some way with this oxidising mechanism.

### PART I.

#### ON THE FUNCTION OF PEROXIDASE IN THE PLANT.

##### 1. *Some observations on the guaiacum reaction.*

If to the solution of a purified peroxidase a few drops of an alcoholic solution of guaiacum and a few drops of 0.01 *N* hydrogen peroxide are added, the immediate development of a blue-green colour proves the activity of the peroxidase. But if the same reagents are applied to the press juice of the plant, which contains almost the whole of its peroxidase, the reaction remains negative, and no colour occurs. This negative result has hitherto generally been attributed to the action of inhibitory substances.

If, for instance, from the roots of the turnip (*Brassica rapa*) a press juice is made, and the above reagents added, no colour appears, although the fluid contains a very active peroxidase which, if reprecipitated with acetone, gives at once a very strong reaction.

If however the quantity of the peroxide added to the crude juice is gradually increased, after a certain quantity of peroxide has been added the green colour suddenly appears and becomes rapidly deeper on the further addition of peroxide. The appearance of this colour depends on the quantity of peroxide added. If a quantity of peroxide is added just insufficient to cause the colour, standing for any length of time does not bring the colour out. Thus the crude juice behaves in the same way as the purified peroxidase, but only after the addition of a certain quantity of peroxide.

This behaviour indicates that the retardation of the reaction in the crude juice is not due to an inhibition in the real sense, but that the first quantities of  $\text{H}_2\text{O}_2$  added are not able to oxidise the guaiacum, because they are used up in another reaction<sup>1</sup>.

The presence of strongly reducing substances in the juice is also indicated by the rapid reduction of iodine. If to the juice starch and then 0.01 *N* iodine are added, the first quantities of the iodine are rapidly reduced. This allows the titration of the juice with fair accuracy with 0.01 *N* iodine. (The end-point of the titration is reached when the reduction of iodine becomes suddenly very slow.)

If, however, peroxide is added before the iodine, the iodine reduction will be found smaller; the decrease of the iodine uptake is equivalent to the peroxide added. The substances which reduce iodine are thus oxidised by the peroxide.

This experiment shows that the first quantities of peroxide, which are unable to colour guaiacum in the crude juice, are used up for the oxidation of a substance which can be oxidised equally well by iodine.

This can clearly be demonstrated if the experiment is made in the reverse order and the iodine is added first, and is followed by the peroxide. In this case the quantity of  $\text{H}_2\text{O}_2$  required for the coloration of guaiacum is diminished by an amount equivalent to the iodine previously added. If sufficient iodine has been added, the first drop of  $\text{H}_2\text{O}_2$  will cause the bluing of the guaiacum. Thus, if the reducing substance is oxidised by iodine, it is unable to use up  $\text{H}_2\text{O}_2$ .

This behaviour of the crude juice may be illustrated by the following experiment.

*Exp.* The root of the turnip is cut up and minced. The juice is pressed out through muslin in a hand press and freed from suspended particles in the centrifuge. The final product is a milky fluid. During the experiment this is kept in large test-tubes so as to reduce its contact with air.

To 5 cc. of the juice a few drops of alcoholic guaiacum solution are added. Then, drop by drop, 0.01 *N*  $\text{H}_2\text{O}_2$  is run in, the fluid being mixed after the addition of every drop. No colour is seen on the addition of the first 1.5 cc. nor does it appear on standing. After addition of 1.6 cc. a green colour appears and becomes rapidly deeper on further addition of  $\text{H}_2\text{O}_2$ .

<sup>1</sup> Catalase could not account for the disappearance of  $\text{H}_2\text{O}_2$ , since the juice shows only a very feeble catalase activity.

To 5 cc. of the above juice 2 drops of strong HCl are added (to inactivate the peroxidase). Then the fluid is titrated with iodine in presence of starch. Uptake 1.9 cc. of 0.01 *N* iodine.

To 5 cc. of the same juice 1.9 cc. of 0.01 *N*  $\text{H}_2\text{O}_2$  are added, the fluid is mixed and after a few seconds acidified with HCl. On titration 0.4 cc. of 0.01 *N* iodine is taken up.

Thus after the addition of  $\text{H}_2\text{O}_2$  1.5 cc. less iodine is used.

To 5 cc. of the same juice 1.9 cc. of 0.01 *N* iodine and then a few drops of guaiacum tincture are added. On addition of the first drops of  $\text{H}_2\text{O}_2$  the guaiacum reaction becomes positive at once.

These experiments thus make it clear that there is in the crude juice a substance (or a set of such substances) which rapidly reduces  $\text{H}_2\text{O}_2$ , and that the first quantities of  $\text{H}_2\text{O}_2$  added are unable to cause the coloration of guaiacum, because they have been used up for the oxidation of this substance.

This substance, which reduces  $\text{H}_2\text{O}_2$  and iodine, also reduces phosphotungstic acid and neutral silver. If to the juice 20 % phosphotungstic acid and then a few drops of ammonia are added, a deep blue coloration shows the reduction of the acid<sup>1</sup>. If to the neutralised juice silver nitrate is added, this is rapidly reduced. Both reductions are absent if  $\text{H}_2\text{O}_2$  has been added previously.

This constituent of the crude juice which reduces the  $\text{H}_2\text{O}_2$  and can be also detected by the reduction of iodine, silver or phosphotungstic acid will be referred to in the following pages as the "reducing factor" (R.F.).

## 2. Mechanism, rate, nature and significance of the oxidation of the R.F. and its distribution.

*Mechanism.* The first question which arises is whether the oxidation of the R.F. by  $\text{H}_2\text{O}_2$  is a direct oxidation, or an oxidation catalysed by the peroxidase. This question can easily be answered by the measurement of the rate of the oxidation in presence and absence of peroxidase. As the following experiment shows, the rate of oxidation of the R.F. by  $\text{H}_2\text{O}_2$  is greatly reduced if the enzyme is inactivated by heating. A similar result is obtained if the enzyme is inactivated by cyanide, so that we can safely conclude that the rapid oxidation of the R.F. by  $\text{H}_2\text{O}_2$  is a peroxidase oxidation.

*Exp.* 5 cc. juice were titrated with 0.01 *N* iodine as in Exp. 1. 5 cc. of the same juice were titrated in the same manner after the addition of 2.0 cc. 0.01 *N*  $\text{H}_2\text{O}_2$ . Iodine uptake in the first = 2.0 cc., in the second = 0.5 cc.

The same experiment was repeated with juice which had previously been rapidly heated to boiling and cooled. Iodine uptake without peroxide = 2.0 cc., with peroxide = 1.9 cc.<sup>2</sup>

<sup>1</sup> Not all phosphotungstic acid preparations could be used for this test. Some preparations gave only very weak colours. Yellow-coloured preparations seemed to give better reactions, but it is not certain whether there is any relation between the yellow colour and the reaction. It is not impossible that the reaction is dependent on some molybdenum compound present as impurity, since the R.F. also strongly reduces phosphomolybdic acid to a highly coloured deep blue product. The phosphotungstic reaction itself is not sensitive to  $\text{H}_2\text{O}_2$ .

<sup>2</sup> If to the boiled juice peroxidase is added, the R.F. is again readily oxidised by  $\text{H}_2\text{O}_2$ .



If the boiled juice is allowed to stand with the peroxide for 5 minutes, and titrated only after this time with iodine, it still takes up 1.0 cc. of the latter.

**Rate.** The most suitable method for following the rate of oxidation of the R.F. is given by the phosphotungstic acid reaction. If a small quantity of juice is added to a solution of phosphotungstic acid, and then a few drops of ammonia added, the developing blue colour indicates the presence and quantity of the unoxidised R.F. This same experiment can be repeated at short intervals after the addition of the  $\text{H}_2\text{O}_2$ .<sup>1</sup> This experiment shows that the oxidation of the R.F. by the  $\text{H}_2\text{O}_2$  is extremely fast. Within 5 seconds the whole factor is quantitatively oxidised, even if the peroxide is added but in slight excess. I was in fact unable to take out the sample quickly enough after the addition of  $\text{H}_2\text{O}_2$  to detect unoxidised reducing substance.

**Exp.** 1 cc. of 20 % phosphotungstic acid is pipetted into each of a series of tubes.

2.5 cc. of turnip juice are titrated with 0.01 *N* iodine. Uptake 0.7 cc.

2.7 cc. of the same juice are put in a test-tube and 0.7 cc. 0.02 *N*  $\text{H}_2\text{O}_2$  are added. Before and immediately after the addition of the  $\text{H}_2\text{O}_2$  a sample of 0.2 cc. is taken and placed in a tube containing phosphotungstic acid.

Then to each tube containing phosphotungstic acid a few drops of strong ammonia are added. On addition of ammonia the first fluid turns deep blue, the second remains colourless.

The next question regards the *nature* of the oxidation of the reducing factor. Is it an irreversible oxidation corresponding to the final oxidation of an intermediary metabolic product, or is it a reversible oxidation corresponding to the oxidation of a catalytic hydrogen carrier?

To decide whether the oxidation is a reversible one, the substance was oxidised by peroxide and peroxidase, and then treated with a mild reducing agent ( $\text{H}_2\text{S}$ ). This experiment clearly showed that the oxidised R.F. can be quantitatively reduced again. The oxidation of the R.F. by  $\text{H}_2\text{O}_2$  and peroxidase is therefore a reversible one.

**Exp.** To 15 cc. of turnip juice 9.3 cc. of 0.01 *N*  $\text{H}_2\text{O}_2$  were added. The negative phosphotungstic acid reaction showed that the R.F. had been oxidised completely. To a second and equal sample 9.3 cc. water were added. The fluid showed the usual strong phosphotungstic acid reaction. Through both fluids a slow stream of  $\text{H}_2\text{S}$  was led for half an hour. Then to both fluids four drops of pure formic acid were added. The fluids were poured into wide beakers. In another beaker was put a strong solution of lead acetate. The beakers were placed over sulphuric acid in the desiccator which was then evacuated. Next morning the volume of both fluids was found to be 15 cc. Both showed by a negative nitroprusside reaction that the  $\text{H}_2\text{S}$  had been removed completely. Both fluids showed an equally strong phosphotungstic acid reaction. To 9 cc. of each fluid 2 cc. of a strong solution of purified peroxidase were added. From each of these two fluids two samples of 5 cc. were taken. One pair of samples

<sup>1</sup> The peroxidase is at once inactivated by the phosphotungstic acid and any further oxidation is stopped.

was titrated with 0.01 *N* iodine (in presence of HCl). Both showed an iodine uptake of 2.3 cc. To the other pair 2.3 cc. 0.01 *N* H<sub>2</sub>O<sub>2</sub> were added, and the fluids titrated with iodine as above. Iodine uptake in both fluids was 0.3 cc.

When it had been shown that the oxidised R.F. could be easily reduced by chemical means, the question arose whether natural oxidising systems were also capable of effecting this reduction. To answer this question the R.F. was oxidised, and the juice incubated.

This experiment showed that reduced R.F. reappears in the juice on standing and that this is due to enzymic activity, since it does not happen in the tube in which the enzymes are destroyed by heating.

*Exp.* 25 cc. of juice were introduced into a Thunberg tube. Then slowly with steady shaking the calculated quantity of 0.01 *N* H<sub>2</sub>O<sub>2</sub> was added (7.5 cc.), which was just sufficient to oxidise the whole amount of R.F. present<sup>1</sup>. The phosphotungstic acid reaction of a sample was negative, or showed only a very slight colour, proving that practically the whole R.F. was oxidised. One part of the fluid was heated in a tube to boiling in order to destroy all enzymic activity, and then rapidly cooled. After boiling the fluid showed a faint phosphotungstic acid reaction. Both portions were put in a Thunberg tube. To both samples toluene was added and both tubes were evacuated and closed. After standing for 4 hours at room temperature the tubes were opened, and the phosphotungstic acid reaction performed. The unboiled fluid showed a strong reaction, the boiled sample showed the same faint colour as immediately after boiling.

*Significance.* It has been shown that the first relatively large quantities of H<sub>2</sub>O<sub>2</sub> added to the juice of the turnip are used up for the oxidation of the so-called reducing factor, and it has been shown that the oxidation of this factor is a reversible one, and that under the given conditions this factor plays the rôle of a catalytic hydrogen carrier between the peroxidase and other oxidising, or reducing, systems. It has also been shown that the rate of the oxidation of this factor is extremely high.

If these experiments can be applied to the intact plant, then the current views on the action of the peroxidase will have to be modified. It is generally assumed that the peroxidase, if supplied by peroxide *in vivo*, would oxidise the most different metabolic products. But, as is known from *in vitro* experiments, the rate of the oxidation of such metabolic products, as for instance lactic acid by peroxide and peroxidase is extremely slow compared to the rate of the oxidation of the above reducing factor. Thus while this factor is present no peroxide will be available for the oxidation of metabolic products, and the only cell constituent oxidised by the peroxidase will be the R.F. which, if oxidised, is reduced again by other systems. The peroxidase will thus have to be looked upon not as an enzyme which oxidises the different metabolic products directly, but as an enzyme having a specific substrate, which,

<sup>1</sup> It is necessary to avoid any excess of H<sub>2</sub>O<sub>2</sub>, because, as Dixon has shown, H<sub>2</sub>O<sub>2</sub> has a very strong inactivating action on certain oxidising enzymes.

playing the rôle of a catalytic hydrogen carrier, connects it up with other oxidising, or reducing, systems.

*Distribution.* The few plants examined in the present research gave the impression that the reducing factor is in its distribution closely connected with the peroxidase. This holds naturally only for the healthy tissue. In ripening fruits, the tissues of which are senescent and colliquating, the proportionality is disturbed through the disappearance of the peroxidase. A strongly developed R.F. has only been found in the so-called "peroxidase plants." The following observations may be noted<sup>1</sup>.

The Spanish black radish (*Rafanus sat. niger*) behaved in the same way as the turnip. Both have a highly active peroxidase, and a relatively highly developed R.F.

The leek (*Allium porrum*) has a still higher peroxidase activity than the turnip. The concentration of the R.F. is also distinctly higher.

The Spanish onion (*Allium cepa*) showed only a very low peroxidase activity and a correspondingly low concentration of the R.F.

In the ripe fruit of the pineapple (*Ananas sativus*) there is a fair concentration of R.F. corresponding to that of the turnip, but the peroxidase is considerably weaker. In the ripe fruit of the tomato (*Solanum lycopersicum*) there is about the same concentration of R.F. as in the turnip or pineapple, but the peroxidase is very weak. Both plants are "peroxidase plants" [Onslow, 1921].

The ripe orange (*Citrus aurantium* Risso) has a high concentration of R.F., about twice that of the turnip. It does not contain any active peroxidase.

The lemon (*Citrus lemonum*) and grape fruit (*Citrus decumana*) have a still higher concentration of R.F. than the orange. Orange and lemon are also typical "peroxidase plants."

Cabbage (*Brassica oleracea*) leaves have also a very high concentration of R.F.; 10 cc. of the press juice correspond to 10 cc. 0.01 N iodine. The peroxidase activity could not be estimated, since sufficient quantity of juice could only be pressed out after the leaves had been exposed to water vapour.

In oxidase plants the R.F., if present at all, seems to be but very weakly developed. 10 cc. of the fresh press juice of the apple reduce 0.3 cc. 0.01 N iodine, while that of the potato reduces 0.9 cc. The corresponding reduction values of the typical peroxidase plants vary in the above experiments between 6 and 10 cc.

<sup>1</sup> The peroxidase activity was examined in the dilute press juice by the guaiacum reaction (in presence of excess of peroxide), and by the rate of oxidation of pyrogallol.

The R.F. was estimated in the press juice by the reduction of iodine, the phosphotungstic acid, reaction and the reduction of neutral silver. In all cases it was also shown that the substance giving these reductions was oxidised at a high rate by peroxide and peroxidase, i.e. that after the addition of peroxide, both reactions became rapidly negative. To the juice of ripening fruits, which contained little or no peroxidase, this enzyme was added.

## PART II.

## OBSERVATIONS ON THE CHEMISTRY OF THE ADRENAL CORTEX.

## THE ISOLATION OF A HEXURONIC ACID.

1. *Evidence of a strongly reducing agent in the adrenal cortex.*

It has been shown in the first part of this paper that in the plant the peroxidase is connected with a strongly reducing substance, or a group of such substances. This factor of the peroxidase system could be recognised by its high reducing power.

To see whether the adrenal cortex contains any such reducing agent the gland was cut with a knife into halves which were immersed in a 0.4 %  $\text{AgNO}_3$  solution. In a few minutes the cortical part was seen to darken through the deposition of reduced metallic silver. In a quarter of an hour the cut surface of the cortex turned completely black, while the medulla remained practically uncoloured (see Plate VI, Fig. 1). The black coloration ends towards the medulla with a sharp margin, which corresponds to the anatomical margin between medulla and cortex. Like the medulla, other organs such as brain, liver and muscle remain practically uncoloured.

If in place of the silver a reagent is used by which adrenaline can be detected, the reverse picture is obtained. If for instance the gland is immersed in 5 % potassium iodate solution, containing 30 % acetic acid, within 30 minutes the medulla turns dark violet, while the cortex remains uncoloured.

The adrenal cortex, therefore, contains some specific, highly reducing agent, which can be no artefact, since its action is produced in the almost fresh material.

If the cortical part is isolated from the medulla and a methyl alcoholic solution prepared from the former, the extract will be found to reduce iodine and phosphotungstic acid in higher degree than can be accounted for by the glutathione present and in much higher degree than the extracts of any other organ. Neutral  $\text{AgNO}_3$  is also reduced at once.

That this reducing agent is a specific constituent of the interrenal system can clearly be demonstrated in elasmobranch fishes (freshly killed *Scyllium canicula*). If the interrenal body is immersed together with another tissue (liver) in 0.2 %  $\text{AgNO}_3$  solution, in a quarter of an hour the interrenal gland is found to be dark, while the liver is unchanged (if not exposed to light).

2. *Isolation of the reducing substance from the adrenal cortex.*

Frozen ox-glands were used which were cut out from the freshly killed animal at the abattoir in London, where they were cooled to  $-6^\circ$ . The glands were transferred to Cambridge in a frozen condition, and kept there at  $-17^\circ$ . No material older than a few weeks was used, since the reducing substance disappears completely in a few months even at this low temperature. The

disappearance is probably due to oxidation, since it begins in the peripheral parts and proceeds towards the centre of the gland.

In the course of purification the reduction of iodine was used as the leading reaction. At a weakly acid reaction the reducing agent can be titrated with a sharp end-point with 0.01 *N* iodine. As soon as the glutathione had been removed, the iodine reduction gave a quantitative measure of the reducing agent, which, compared with the weight of the dry residue, gave a measure of the purity.

The following properties of the substance were of use in the final method of isolation.

*Solubility.* In all preliminary experiments the substance behaved as an acid without basic groups.

The free acid is exceedingly soluble in water, freely soluble in methyl alcohol, less readily soluble in ethyl alcohol and acetone, slightly soluble in butyl or amyl alcohol, insoluble in ether or light petroleum. From its alcoholic solution—if not in very high concentration—it is not precipitated by ether, but is precipitated to a great extent by light petroleum. It is precipitated nearly completely from acetone by light petroleum.

*Sodium, barium and ammonium salts.* The sodium and ammonium salts are freely soluble in methyl alcohol, less readily soluble in ethyl alcohol, slightly soluble in acetone, insoluble in ether. Both salts are precipitated from alcoholic solution by ether. Still smaller is the solubility of the barium salt in the above solvents. All three salts are soluble in water.

*Lead salts.* The substance forms two different salts with lead. With neutral lead acetate it forms a compound, soluble in water, insoluble in alcohol. It is necessary to employ an excess of lead to obtain a quantitative precipitation.

The substance is precipitated by basic lead acetate equally from water and alcohol.

*Stability.* In absence of oxygen the substance is stable in water, is not destroyed by short boiling, and can be treated with dilute mineral acid. In anhydrous solvents, such as alcohol or acetone, it is extremely sensitive to mineral acid, and resinifies readily under their influence. Even strong organic acids, such as picric acid, effect its resinification.

The substance is highly autoxidisable. It is oxidised irreversibly by molecular oxygen. The oxidation is greatly catalysed by OH ions, and heavy metals, such as copper. It is inhibited by cyanide.

The substance is not fermented by yeast.

*Crystallisation.* The free substance does not crystallise under any conditions, but forms an anhydride, which readily crystallises, and can be easily recrystallised. Dissolved in water this readily adds water to form the acid.

*Method of isolation.* For the extraction of every kg. of glands three litres of methyl alcohol are used. To every litre of the alcohol 0.1 cc. 10 % NaCN is added. CO<sub>2</sub> is led through the alcohol to remove oxygen, and the alcohol is cooled on ice.

The glands are minced in a frozen condition to a fine pulp which is immediately mixed with alcohol. The suspension is allowed to stand for half an hour, being stirred frequently by a strong current of  $\text{CO}_2$ . Then with vigorous stirring a 50 % solution of barium acetate is added sufficient to make the final concentration 0.5 %.

After standing on ice for another half hour and with occasional stirring, the suspension is rapidly filtered through muslin, the residue being pressed out in a hand press. The fluid is rapidly filtered through paper pulp and the clear filtrate is transferred to bottles. These are kept on ice, the air in the bottles being replaced by  $\text{CO}_2$ .

A hot saturated solution of lead acetate is added, sufficient to bring the final concentration to 5 %. The fluid is allowed to stand on ice for another hour and is then filtered on a Büchner funnel, the paper in which has been covered with a thin sheet of fine asbestos. The precipitate is washed with methyl alcohol<sup>1</sup>.

The precipitate is suspended in as small a volume of water as possible and decomposed by sulphuric acid. The acid is added as 20 % solution in small quantities with stirring and ice-cooling, excess being avoided. The lead sulphate is filtered off. If the filtrate colours thymol blue, sodium bicarbonate is added till the coloration disappears, then the fluid is reduced at 20–30° *in vacuo* to small volume and dried in a vacuum desiccator over sulphuric acid. To absorb acid vapour filter-paper soaked in strong NaOH solution is placed in the desiccator.

The dry residue is extracted with a small quantity of absolute methyl alcohol. To the methyl alcohol five times its volume of freshly distilled water-free ether is added. The precipitate is separated and added to the insoluble residue, which is then extracted again with a small quantity of methyl alcohol, to which again ether is added. The clear alcohol-ether fluids are united. In this way the residue corresponding to 1 kg. of the gland is extracted three times with 10 cc. of methyl alcohol.

The alcohol-ether is cooled in ice, and dry ammonia gas is passed in until the fluid contains an excess of ammonia. Then the fluid is filtered and the precipitate dissolved in a small quantity of water, to which a sufficient quantity of acetic acid has been added to make the final reaction acid. For 1 kg. of the gland 50 cc. of dilute acetic acid solution are used.

After the precipitate has been dissolved the fluid is neutralised by the addition of ammonia, and then 20 % of lead acetate is added in excess in the form of a hot saturated solution. If any precipitate is formed, this is separated on the centrifuge, and then to every 50 cc. of the fluid 150 cc. 96 % ethyl alcohol is added. The fluid is cooled for an hour on ice, and then filtered.

The lead precipitate is dried over sulphuric acid *in vacuo*.

The dry precipitate is powdered and suspended in water-free, freshly

<sup>1</sup> If more convenient, this precipitate can be placed over sulphuric acid in the vacuum desiccator, and kept there for a few days.

distilled acetone, for every kg. of the gland about 100 cc. acetone being used. Then  $H_2S$  is passed through until the whole precipitate is decomposed, the acetone is then evaporated at low temperature *in vacuo* to a very small volume. To this an equal amount of ether and an excess of light petroleum are added, which precipitate an oily material, and keep most of the acetic acid in solution. The oily precipitate is transferred to an Erlenmeyer flask (the distilling flask being washed out with a few cc. of methyl alcohol). Here the syrup is dried again over sulphuric acid *in vacuo*, NaOH paper being placed in the desiccator.

The solid residue is extracted repeatedly with small volumes (5 cc.) of water-free acetone, which is then evaporated *in vacuo* at low temperature. The residue is dissolved in a very small volume (2-3 cc.) of methyl alcohol. To this 15 cc. of water-free ether are added. If an amorphous precipitate forms, it is separated and extracted with acetone and the acetone treated as above.

To the clear alcohol-ether an excess of light petroleum is added (10 times the volume of the fluid), and the mixture placed in the ice-chest.

The next morning a crystalline precipitate is found on the bottom of the flask with some oily material. The crystals are freed from the oil by washing with a small amount of ice-cooled acetone. Water-free ether is used to wash the crystals out of the flask and remove the last trace of acetone.

The main impurity of the preparation in the last stages are resinification products of the substance itself. These are less soluble in acetone and alcohol-ether than the unchanged substance.

The yield of the preparations, working with quantities of 1-3 kg. of the gland, is 300 mg. per kg. This corresponds approximately to half the total amount present in the gland.

The dried crystals are stable *in vacuo*, and decompose slowly in the air.

### 3. *The chemical nature of the reducing substance.*

The crystalline preparation can be recrystallised most conveniently by dissolving the crystals in a small volume of absolute methyl alcohol, and gradually adding ether and light petroleum.

In presence of water the substance does not crystallise but forms a sticky syrup. This syrup, however, on standing *in vacuo* over sulphuric acid slowly loses water and solidifies to a crystalline mass.

The substance is polymorphic. The primary crystalline preparation usually consists of small plates, which combine to more complicated figures (Plate VI, Fig. 2 a). Recrystallised from acetone with ether the same crystals, or needles, may be obtained (Fig. 2 b). Different structures are obtained by evaporating a methyl alcoholic solution on an object slide (Fig. 2 c), a watery solution in a thin layer (Fig. 2 d), or in a larger quantity (Fig. 2 e).

The preparations of the substance show a fairly sharp melting-point, ranging between 175 and 189°. A few degrees below the m.p. the substance turns first yellow and then brown, and after melting forms a brown resin.

The M.P. of my successive preparations were: 175, 183, 175, 186, 186, 189, 185, 182°.

*Molecular weight.* The minimum molecular weight could be determined by two different methods, by acidimetric and by iodometric titration.

4.993 mg. of the substance were dissolved in 3 cc. of water. Titration in presence of phenolphthalein with 0.0216 *N* NaOH required 1.51 cc. Thus the equivalent weight -- 178.

After the titration had been finished, one drop each of 33 % acetic acid and starch solution was added, and the mixture was titrated with 0.01 *N* iodine, requiring 5.66 cc. corresponding to an equivalent weight of 88.7.

The experiment shows that the minimum molecular weight given by the acidimetric titration is 178, and that for each carboxyl group two atoms of iodine are reduced.

The molecular weight was estimated by the method of Barger.

9 mg. of the substance were dissolved in 0.5 cc. of water. This was sealed in capillaries with 0.4, 0.2, 0.1 and 0.05 *M* urea solutions. The tubes were left for 24 hours at 37°. In the tubes 1 and 2 the urea solution was hypertonic. No movement of the meniscus occurred in tube 3; in tube 4 the urea was hypotonic; whence m.w. = 178 ( $\pm 2$  %).

*Optical activity.* 30 mg. of the substance were dissolved in 1.5 cc. of water: temp. 21°. The rotation was : 0.48° in a 1 dm. tube. This corresponds to  $[\alpha]_D^{21} = +24^\circ$ . The substance showed no mutarotation. (Observed for a week.)

*Elementary analysis.* The elementary analysis was made by "Feinchemie, Tübingen." In the qualitative analysis the substance shows no N, Cl, P, S, or OMe.

4.600 mg. gave 6.865 mg. CO<sub>2</sub> and 1.97 mg. H<sub>2</sub>O.

	Found	Calc. for C <sub>8</sub> H <sub>8</sub> O <sub>8</sub> (m.w. 176)
C	40.7	40.9
H	4.7	4.5
O	54.6	54.6

*Chemical reactions.* Heated in dry condition the substance resinifies, giving a strong smell of caramel.

Fehling's solution, alkaline or neutral silver and permanganate are readily reduced at room temperature.

The Molisch reaction is strongly positive with a purple colour.

The orcinol test (with Fe) is also strongly positive.

The substance gives thus all the typical carbohydrate reactions tested.

*Constitution.* The above data make it clear, that the reducing substance is a highly reactive carbohydrate derivative, isomeric with glycuronic acid,

The accuracy of the reading of optical activity lies within 10 %.



which, by losing water, goes over reversibly into the crystalline lactone anhydride, corresponding to the formula  $C_6H_8O_6$ .

No attempt was made in the present research to elucidate the steric configuration of the molecule. In the latter part of the paper some arguments are given in favour of the view that the substance is a hexuronic acid derived from fructose.

The substance seems not to be identical with any of the glycuronic acid isomers hitherto described.

The high reactivity excludes identity with glycuronic acid. The solubility of the calcium salt and the different optical activity excludes the identity with the acid investigated by Bertrand [1904], Boutron [1886], Ruff [1899] and Kiliani [1922]. The high reactivity and the different melting-point and optical activity exclude the identity with the galactosuric acid, isolated from plants by Ehrlich and Sommerfeld [1925].

Since the exact constitution of the reducing substance is unknown, I propose to refer to it as a hexuronic acid.

A few words may be said about the specificity of this substance for the adrenal cortex.

As has been shown above, the substance is specific for the cortex in as far as no other animal organ contains it in amounts comparable to those present in the interrenal tissue. But whether other tissues contain it in small quantities, or whether the substance is absent from other organs altogether, cannot be stated at present. The extracts of all organs have some reducing action, 8–20 times smaller than that of the adrenal cortex. How far this reduction is due to hexuronic acid, or to other substances, cannot be stated, since the quantities possibly present are too small for isolation, which is the only reliable way of identification. As will be shown in a later part of this paper, the hexuronic acid is extremely active as a catalyst in certain systems of oxidation-reduction. Concentrations of 1 : 0.000025 are sufficient to show a distinct biological activity, so that this substance may play an important rôle in the oxidation mechanism of all animal tissues.

## PART III.

ISOLATION OF THE HEXURONIC ACID FROM PLANTS AND SOME OBSERVATIONS  
ON THE CHEMISTRY OF THE REDUCING FACTOR.1. *The isolation of the hexuronic acid from plants.*

After the study of the reducing factor had led to the isolation of a hexuronic acid from the adrenal cortex, the question arose whether this acid actually occurs among the reducing substances of the plant. This question could be answered only by the isolation of the acid.

Before the method used for the adrenal could be applied, the water of the plant juice had to be eliminated, and the bulk of accompanying substances reduced. A great number of accompanying impurities could be eliminated by precipitation with barium and alcohol. The water could be eliminated by vacuum distillation. The most effective step in the purification was the precipitation of the acid by lead from its alcoholic solution at slightly acid reaction. This precipitation is however inhibited in the crude juice, and it was necessary to precipitate the substance first with basic lead to eliminate the inhibitory factor.

After this preliminary treatment the original adrenal method was adopted almost unchanged, and led to the isolation of a substance identical with hexuronic acid. This substance, which forms in the animal a specific constituent of the adrenal cortex, seems to be widely distributed in the vegetable kingdom, forming an essential constituent of the reducing substances, apparently connected with the function of the peroxidase system.

As starting material for the isolation, orange juice and the watery extract of cabbages were chosen.

2. *Isolation of the hexuronic acid from oranges.*

*Method.* The ripe fruit is cut in two, and squeezed out on a lemon squeezer. The juice is filtered through muslin. To every litre 0.1 cc. of 10 % sodium cyanide is added. By addition of ammonia the fluid is then brought to  $p_H$  6.2, and 5 % barium acetate is added in the form of a hot saturated solution. The fluid is left in full closed bottles overnight in the ice-chest and filtered the next morning through paper pulp. The filtrate is concentrated *in vacuo* at 25° to one-fifth of its volume. The concentrated fluid is cooled in ice and filtered from the newly formed precipitate. Again sodium cyanide (0.1 cc. 10 % to 1 litre) and then hot saturated lead acetate are added, sufficient to make up the total concentration to 10 %. The fluid is cooled in ice, then with vigorous stirring ammonia is added until the fluid just colours bromothymol blue. The fluid is quickly filtered, the precipitate washed with water and suspended in the smallest possible volume of water and decomposed with sulphuric acid, excess being avoided. After the lead sulphate is removed by

filtration, the  $p_H$  is adjusted to 6. If necessary, the volume of the fluid is reduced by vacuum distillation to one-twentieth of the original bulk. Three times its volume of 96 % alcohol is then added, the precipitate, if formed, separated and the fluid mixed with excess of hot saturated lead acetate. The fluid is cooled on ice for an hour and filtered. The precipitate is treated further by the method described in the first part of this paper.

The crystalline precipitate obtained consists of small irregular crystals. Recrystallised these show the same polymorphism as the substance isolated from the adrenal gland. The m.p. in the different preparations was 182, 178 and 175°.

The acidimetric titration gives an equivalent weight of 180 ( $\pm 2$  %). The more accurate iodometric titration gives an equivalent weight of 89. Thus for each acid group two atoms of iodine are reduced and the minimum m.w. given by the titration is 178 g.

The estimation by Barger's method gives m.w. 178.

The substance has  $[\alpha]_D^{20} + 24^\circ$ , and displays no mutarotation.

Analysis (by "Feinchemie Tübingen") gives theoretical values for  $C_6H_8O_6$ .

	Found	Calc. for $C_6H_8O_6$
	%	
C	40.9	40.9
H	4.5	4.5
O	54.6	54.6

All chemical reactions were identical with those found with the acid from the adrenal cortex.

These data sufficiently establish the identity of the substances isolated from the adrenal cortex and from orange juice. Identical substances were obtained from the orange juice in three subsequent preparations. Working with 1.5–3 litres of juice the yield is about 100 mg. per litre.

#### *Isolation of the hexuronic acid from cabbages.*

The isolation of the acid from cabbages is made still more difficult by the accompanying substances.

The fresh leaves are immersed in 1 % acetic acid, for each kg. of the plant 1 litre being used. Then the fluid is heated to 80°, cooled, and pressed out in a mechanical press. To every litre 0.1 cc. 10 % NaCN is added. Sufficient barium acetate is then dissolved in the fluid to make it up to 1 %. Then a hot saturated solution of lead acetate is added, sufficient to make the final concentration 7.5 %. The precipitate is separated and the fluid cooled in ice, made alkaline with ammonia (to bromothymol blue) and filtered. The precipitate is treated in the same way as that from the oranges.

From 5 litres of juice 250 mg. crystalline matter were obtained, of the same crystal structure as the substance from oranges; m.p. 188°. In all reactions this substance behaves in the same manner as that isolated from oranges.

The method does not seem to work on a larger scale with this material. Two attempts to isolate the acid from 300 kg. and 50 kg. were fruitless.

### 3. *The chemistry of the reducing factor.*

It has been shown in the first part of this paper that 100 cc. of orange juice reduce about 60 cc. of 0.01 *N* iodine. This would correspond to about 50 mg. hexuronic acid anhydride and about 10 mg. of this substance can actually be isolated in crystals, corresponding to 11 cc. of 0.01 *N* iodine. Since the preparation is accompanied by great losses it is clear that this compound must form an essential part of the "reducing factor." But the reagents employed for the demonstration of the R.F. are not specific, so we can hardly expect that the whole R.F. should be constituted by the hexuronic acid. Phosphotungstic acid and silver are reduced by most phenolic substances, and iodine is reduced by many phenols. Thus we can expect that the phenols present would constitute a part of the R.F. if the above reagents are employed. In fact there is present in orange juice, as in cabbage juice, a phenolic substance in rather high concentration, which reduces all three of the above reagents, so that about half of the R.F. must be composed of this phenolic substance.

The reducing properties of plant juice have repeatedly attracted attention, specially from students of vitamin C. Bezssonoff [1921] has applied Folin's phosphomolybdic acid reagent. The reducing substances of lemon juice have been made the object of a thorough study by Zilva [1927, 1928], who established interesting relations between vitamin C and the reducing properties of the plant juice. The main reagent employed by Zilva [1928] was phenol-indophenol. Indophenol blue is readily reduced by the hexuronic acid, so that it is probable that it was this substance which has been studied by Zilva. Phenols are less likely to reduce this indicator.

## PART IV.

### OBSERVATIONS ON THE OXIDATION AND REDUCTION OF THE HEXURONIC ACID.

#### 1. *The reduction potential of the hexuronic acid.*

A 1 % solution of the acid was neutralised with 0.1 *N* NaOH, then phosphate buffer ( $p_H$  7) was added and the mixture diluted with water. The final concentration of the acid was 0.2 %, that of the buffer 0.01 *M*.

This solution has been added to the series of Clark indicators. Nos. 1, 2 and 3 were not reduced. No. 4 was reduced fairly rapidly. No. 8 was readily reduced. Methylene blue was reduced slowly to completion.

The preliminary measurement of the potential with the gold electrode showed that the substance has a definite reduction potential and decreases the  $E_H$  of the pure buffer solution from + 240 mv. to + 90 mv., corresponding to an  $r_H$  of 17.14.

The hexuronic acid shows thus, like glutathione and thyroxine, a distinct and fairly high reduction potential.

I am indebted to Dr M. Dixon for his kind assistance in these measurements.

### 2. *Oxidation by iodine.*

A small quantity of the acid is dissolved in water; 1 cc. of the solution reduces 1.3 cc. 0.01 *N* iodine.

Three further samples of 1 cc. are taken. To each one drop of starch is added. To the first sample an alcoholic solution of iodine is added, till the first persistent blue colour appears. The excess of iodine is reduced by the addition of a trace of the acid. Then to each of the samples thymol blue is added. The first sample which has been oxidised is acid, the others alkaline to thymol blue. Then to the two samples which have not been oxidised 0.01 *N* HCl is added until they reach the colour of the first sample, 1.26 cc. and 1.23 cc. being required.

Thus for each atom of iodine added 1 mol. of HI was formed, the oxidation of hexuronic acid consisting therefore in the removal of two H atoms.

### 3. *Oxidation by molecular oxygen.*

The solution used in the potential measurement is placed in the Barcroft apparatus and shaken at room temperature. 4 mm.<sup>3</sup> O<sub>2</sub> are taken up per minute.

After 10 minutes the apparatus is opened and to the fluid 1 cc. of 0.01 *M* neutralised KCN added. No further oxygen is taken up on shaking at room temperature.

This experiment shows that, corresponding with its reducing potential, the substance is fairly autoxidisable. As the autoxidation is inhibited by cyanide, it must be a heavy metal catalysis.

The following experiment was made on the nature of the heavy metal.

To a 0.01 *N* (iodometric) solution of the hexuronic acid a double volume of 0.1 *M* phosphate buffer of  $p_H$  7 was added. The oxygen uptake of 3 cc. of this fluid was measured in the Barcroft apparatus, 3 cc. of water being used in the compensating flask. Then the oxygen uptake of 3 cc. of the same fluid was measured in presence of 0.1 cc. 0.005 *N* FeCl<sub>3</sub>, CuCl<sub>2</sub> and manganese acetate. Without metal, or in the presence of Fe or Mn, the acid showed no measurable oxygen uptake. In presence of copper the fluid showed an intense uptake of oxygen (20 mm.<sup>3</sup> per minute). In 15 minutes the oxidation of the acid is complete. The autoxidation of the hexuronic acid is thus greatly catalysed by copper, but it is not catalysed, or catalysed in much smaller degree, by iron and manganese.

Since the acid is readily oxidised by trivalent iron, the inactivity of this substance as a catalyst gives evidence against those theories which explain the catalytic action of iron by its reduction by the autoxidisable substance and its subsequent autoxidation.

The oxidation of the acid by H<sub>2</sub>O<sub>2</sub> is also greatly catalysed by copper.

#### 4. *The chemical nature of the oxidation products.*

If the hexuronic acid is oxidised by iodine or silver, it can be reduced again quantitatively by reducing agents such as  $\text{H}_2\text{S}$ . The two atoms of H which are given off on oxidation can thus be again replaced.

If Fehling's solution is added to the unoxidised acid, the reagent is readily reduced at room temperature. After a while the reduction stops, but proceeds again on boiling. But if the acid is oxidised first by iodine or silver, Fehling's solution is not reduced at room temperature. If however the oxidised acid be heated with Fehling's solution the reagents are readily reduced. The Tollens silver reagent shows an analogous behaviour. This shows that the aldehyde or ketone function of the acid is not involved when the substance is oxidised at room temperature by iodine, silver or Fehling's solution. It is thus not this function to which the high reducing power is due.

If the substance is oxidised by molecular oxygen at neutral reaction, copper being used as catalyst, the oxidation product which is unable to reduce iodine, neutral silver, or Fehling's solution at room temperature still reduces the latter reagent on boiling. Thus the aldehyde or ketone function is not involved in the autoxidation. But if the acid is oxidised by oxygen, the oxidation product is irreversible, and cannot be reduced again by  $\text{H}_2\text{S}$ . If the fluid is titrated with alkali, it is found that new acid equivalents are formed on autoxidation<sup>1</sup>. The oxidation of the two labile hydrogens leads thus to the formation of carboxyl groups, which makes it very probable, that it is a terminal carbon atom which carries the two labile hydrogen atoms. The lability of these hydrogen atoms makes it probable that their carbon atom is next to the carbonyl group. This would correspond to the structure of a fructuronic acid, which can be expected to have a  $\gamma$ -lactone oxygen since it shows no mutarotation.

#### 5. *The oxidation by peroxidase and peroxide.*

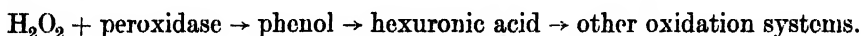
It has been shown in the first part of this paper that in the plant juice the hexuronic acid is readily oxidised by peroxide and peroxidase.

After the substance had been isolated, purified peroxidase and peroxide were added to its solution, in order to study this oxidation. The experiment gave the unexpected result that purified peroxidase had no effect on solutions of the crystalline compound. If to its solution some plant juice (cabbage or orange) was added, together with the peroxide and peroxidase, it was oxidised at a high rate. This made it clear that the peroxidase has no direct action on the acid, and the rapid oxidation in the plant juice is due to the presence of catalytic substances.

<sup>1</sup> Corresponding with the high rate of autoxidation the new carboxyl groups are formed rapidly. Every molecule of hexuronic acid forms one new carboxyl group. When these new carboxyl groups have been established, the substance goes on forming new acid groups, but at a very low rate. This further formation of acid groups is probably due to the breaking up of the molecule.

Much time was given to the study of the nature and action of these catalysts. The details of this extended study will not be given here to avoid a further extension of the present paper. It was found that the catalysts of this reaction are aromatic substances which play the rôle of a catalytic hydrogen carrier between the acid and the peroxide or peroxidase. The phenolic substances are in this reaction oxidised to quinones which in their turn oxidise the labile hydrogen of the acid, being themselves reduced again to their original phenolic form. Thus any phenol which is oxidised by peroxide and peroxidase will be able to catalyse the reaction between peroxide and peroxidase on the one side, and hexuronic acid on the other.

In the plant, therefore, there are two catalytic hydrogen carriers between the peroxidase and oxidation systems, in which the hydrogen of the foodstuffs is activated. The scheme of oxidation is thus the following:



#### 6. *The oxidation in coupled systems.*

What the source of the peroxide is in the living tissue and where it is generated, are as yet unknown. Different oxidation systems are known, which are normal cell constituents and which lead to the formation of peroxide. Wieland and Sutter [1928] have shown that the thermostable oxidase, acting on quinol produces hydrogen peroxide in equivalent amount to the oxygen adsorbed. Thurlow [1925] has shown that the oxidation of acetaldehyde by the Schardinger enzyme is connected with the formation of peroxide.

We may expect that the  $\text{H}_2\text{O}_2$  formed in any of these or other systems can be used for the oxidation of the hexuronic acid, and it seemed worth while to demonstrate that the  $\text{H}_2\text{O}_2$  formed in such an oxidation can actually be used for the oxidation of this component.

The Schardinger enzyme was used as material for this experiment. It might be expected that in the presence of  $\text{O}_2$  and acetaldehyde the enzyme would generate  $\text{H}_2\text{O}_2$ , which, in presence of peroxidase, would act on an aromatic substance which in its turn would oxidise the compound. It would be expected that the oxidation of the phenol would reveal itself in the formation of the coloured quinone, and that in presence of hexuronic acid the solution would remain uncoloured, owing to the reduction of the quinone by the acid.

To 1000 cc. of fresh milk 10 cc. rennin were added, the milk was warmed to  $37^\circ$ , and the whey separated by filtration through muslin. To the whey an equal volume of saturated ammonium sulphate was added, the fluid filtered and the precipitate washed with half saturated ammonium sulphate. The precipitate was dissolved in 150 cc. water, precipitated with 150 cc. ammonium sulphate and washed as above. The precipitate was extracted with ether, dissolved in 150 cc. of water, once more precipitated with ammonium sulphate, and finally dissolved in 300 cc. 0.1 *M* phosphate buffer of  $p_{\text{H}}$  7.

2 cc. of this solution *in vacuo* in presence of 0.02 cc. of acetaldehyde

reduces 0.1 cc. 0.1 % methylene blue in 90 seconds at room temperature and in 15 seconds at 37°.

On addition of peroxide and peroxidase the fluid slowly develops a faint blue colour, showing that it contains but little peroxidase.

In all experiments 2 cc. enzyme, 0.02 cc. acetaldehyde, 0.2 cc. 0.1 % adrenaline and 0.1 cc. 1 % peroxidase (turnip) were used.

2 cc. of the enzyme solution were placed in a test-tube, aldehyde, peroxidase and adrenaline added, the tube was immersed in water at 37° and aerated. Within one minute a strong red colour indicated that the adrenaline had been partly oxidised to quinone. It is easy to prove that the adrenaline has in fact been oxidised by the peroxide formed in the oxidation of the aldehyde. If the aldehyde or the Schardinger enzyme are omitted, or if the Schardinger enzyme is inactivated by boiling, no coloration occurs. If the peroxidase is left out, only a very faint colour occurs (owing to the slight amount of milk-peroxidase present). Also in presence of cyanide the coloration remains very faint. The experiment gives very clear results and gives a very striking and simple demonstration of the formation of  $H_2O_2$  in biological oxidation.

If the hexuronic acid is added to the same system, the mixture remains uncoloured as long as there is unoxidised acid present. As soon as the acid is used up, the red colour appears. Thus the appearance of the red colour coincides with the disappearance of reduced hexuronic acid, judged by the phosphotungstic reaction. If to the above system 0.05 mg. of hexuronic acid is added, the fluid remains uncoloured for one minute. With 0.1 mg. it remains uncoloured for two minutes, with 0.2 mg. for 4 minutes, with 0.4 mg. no colour appears at all during the 9 minutes of observation.

Thus even a concentration of 0.0025 % of the acid is sufficient to prevent the formation of the pigment, *i.e.* to reduce the quinone formed, as long as the acid is present in reduced condition. Since tissues, as has been shown in the first part and will be shown again later, energetically reduce oxidised hexuronic acid, it can be expected that in the cell the oxidised acid will be reduced again, and in this way be maintained in reduced condition. In this case the concentration of 0.0025 % will be sufficient to show a distinct biological activity.

This experiment deserves perhaps a special consideration since it shows that, in a system composed of normal cell constituents, the formation of pigment can be prevented by a specific constituent of the adrenal cortex. It may be remembered that one of the best known symptoms of a deficiency of the interrenal system is the formation of iron-free pigment.

#### 6. *Oxidation by the indophenoloxidase.*

As is known from the work of Battelli and Stern [1912] washed muscle contains a very active enzyme, capable of oxidising *p*-phenylenediamine at a high rate. The recent experiments of Keilin [1929] make it probable that this enzyme plays a very important part in tissue respiration.

It was found that this enzyme is unable to oxidise the hexuronic acid. If muscle is washed and shaken in the Barcroft apparatus with a solution of



the acid, no oxygen uptake is observed and the hexuronic acid is found un-oxidised at the end of the experiment.

It may also be noted that the reduction of methylene blue by the hexuronic acid is not catalysed by muscle. Thus the hydrogen of the acid is not activated by tissues, which makes it probable that the substance cannot be oxidised definitely and used as foodstuff by the animal cell.

### 7. Oxidation by haematin compounds.

The hexuronic acid reduces haematin compounds, but the rate of reduction is too slow to indicate that the acid is actually connected in its function with these respiratory pigments.

Experiments were made with cathaemoglobin and cytochrome. Cathaemoglobin is reduced by small amounts of hexuronic acid *in vacuo* overnight. No effect can be observed in a short experiment.

A solution of cytochrome C is reduced in a few minutes by small quantities of the acid. The cytochrome of washed muscle is reduced in about 5 minutes. The cytochrome of the washed muscle is reduced by hexuronic acid about 30 times more slowly than by succinate (which is acted on by the succinodehydrase).

The oxidation of the hexuronic acid by hydrogen peroxide is greatly catalysed by haematin compounds (cathaemoglobin). The concentration of hydrogen peroxide which is necessary to effect a rapid oxidation of the acid is much too high to assume that the haematin would act *in vivo* as peroxidases on hexuronic acid.

I am indebted to Dr D. Keilin for his kind assistance in these experiments.

### 8. The reduction of oxidised hexuronic acid by animal tissues.

If an animal tissue, such as kidney, liver or muscle, is minced and suspended in a little buffer solution, and oxidised hexuronic acid is then added and the suspension incubated for a short time, for instance 15 seconds, the hexuronic acid will be found to be partly present again in reduced form at the end of the incubation.

*Exp.* The kidneys (1.8 g.) of a freshly killed rat are cut into pieces, and then minced in a mortar with a little sand. 2 cc. 0.2 *M* phosphate buffer of  $p_H$  7 is added, and the suspension divided into two equal parts. To one part 0.5 cc. water is added, to the other 0.5 cc. oxidised 0.01 *M* hexuronic acid (oxidised with iodine). The suspensions are incubated for 15 minutes in a water-bath. Then equal parts of 6 % trichloroacetic acid are added to both. The samples are centrifuged. The fluids are tested with phosphotungstic acid for reduced hexuronic acid and with sodium nitroprusside for glutathione. A portion of both samples is neutralised with  $\text{NaHCO}_3$ , and silver nitrate added. The fluid with hexuronic acid gives a weaker —SH reaction with nitroprusside, but a much stronger reaction with phosphotungstic acid, and reduces silver nitrate.

Two oxidising systems are known in animal tissues which might be expected to reduce oxidised hexuronic acid: the Thunberg-Wieland hydrogen-activating system, and the Hopkins glutathione system.

#### 9. *The Thunberg-Wieland system.*

Two main types of enzymes are known in this system: the type of the Schardinger enzyme system and the type of the succinoxidase. The former are soluble, and consist only of a hydrogen activator, the latter are insoluble and consist of a hydrogen activator and an oxygen activator [Fleisch, 1924; Szent-Györgyi, 1927, 3].

*The Schardinger enzyme.* This enzyme appeared to be unable to reduce oxidised hexuronic acid. The following experiment may be noted.

*Exp.* 0.2 % hexuronic acid is oxidised with 0.1 *N* iodine-KI solution. To 4 cc. of the above enzyme solution 0.04 cc. acetaldehyde and 0.4 cc. of the oxidised acid are added. The phosphotungstic acid reaction after 10 minutes' incubation is negative.

The *succinodehydrase* was also found to be inactive.

*Exp.* Ox diaphragm muscle was minced and washed three times with 20 times its own volume of distilled water, shaking it for 15 minutes. The muscle was filtered through muslin and squeezed out.

2 g. of this muscle in 10 cc. buffer (0.2 *M* phosphate of  $p_H$  7) in presence of 0.1 % succinate reduces 0.4 mg. methylene blue in 9 minutes.

1 g. of this muscle is suspended in 2 cc. phosphate, then 0.25 cc. 2 % sodium succinate and 0.5 cc. 0.2 % oxidised hexuronic acid are added. The suspension is incubated in evacuated Thunberg tubes for 35 minutes. In control experiments the succinate, the muscle and the hexuronic acid are replaced by water.

After the incubation the tubes are cooled in ice, opened, and the liquid tested with phosphotungstic acid.

The phosphotungstic acid reaction is negative in the tubes containing no muscle, positive in the tubes with muscle. Thus in the presence of muscle a small part of the oxidised hexuronic acid has been reduced. But, since the reaction is equally strong in tubes with and without succinic acid, the reduction is not due to the succinodehydrase.

The reduction of methylene blue by muscle and succinate is not inhibited by oxidised hexuronic acid. This substance therefore does not inhibit the dehydrase.

#### 10. *Reduction by the Hopkins glutathione system.*

Having found that the Thunberg-Wieland dehydrases do not reduce the substance it seemed probable that it is the Hopkins system to which the reduction is due. This system consists of glutathione, fixed SH-groups and a hitherto unknown factor, reducing the glutathione, either directly or through the fixed SH-groups.

*Reduction by fixed SH-groups.* It has been shown in the last experiment that the washed muscle reduces oxidised hexuronic acid. The experiment was completed by carrying out the nitroprusside reaction with the muscle after its incubation with oxidised hexuronic acid. The experiment showed that the

reaction for fixed SH-groups became much weaker if the muscle was incubated with the oxidised acid. This shows that the acid is reduced by fixed SH-groups.

*Reduction by glutathione.* If oxidised hexuronic acid is incubated at neutral reaction with reduced glutathione, the former becomes reduced, the latter oxidised.

*Exp.* A 0.02 *N* (iodometric) solution of reduced glutathione and a 0.06 *N* solution of hexuronic acid are prepared. The latter is oxidised with a strong alcoholic solution of iodine. Both solutions are neutralised with 0.1 *N* NaOH. To both one-tenth part of 0.2 *M* phosphate buffer ( $p_H$  7) is added. Equal parts of both fluids are mixed and incubated. The quantity of SH is roughly measured by the intensity of the nitroprusside reaction. The experiment shows that in 2½ minutes about one-third, in 5 minutes about two-fifths, and in 10 minutes about half of the glutathione is oxidised. (The phosphotungstic reaction cannot be used in this experiment, since reduced glutathione also reduces phosphotungstic acid, though in much smaller degree than the hexuronic acid.) After incubation *in vacuo* for 90 minutes the —SH reaction reveals only a trace of reduced —SH, showing that the oxidation of —SH is practically complete.

In control experiments, made without phosphate buffer, parallel to the disappearance of the nitroprusside reaction, the silver reduction, which is absent at the beginning, becomes gradually stronger.

The —SH reaction of neutral glutathione does not change under identical conditions, if no oxidised hexuronic acid is present.

The experiment thus shows that glutathione reduces the oxidised acid fairly rapidly. The fixed —SH is also able to reduce the acid. Whether the third factor of the system, which reduces the S—S link, also directly reduces the oxidised acid, or only through the labile and fixed —SH, cannot be stated at present.

#### SUMMARY.

*Part I.* If  $H_2O_2$  is added to the juice of a peroxidase plant, the first quantity of the peroxide is used up for the oxidation of a certain substance, or a group of substances, referred to as the "reducing factor" (R.F.). This oxidation of the R.F. is greatly catalysed by the peroxidase. The rate of the oxidation of this factor is very high, and as long as this factor is present no other substances will be oxidised by the peroxidase, and peroxide reactions such as the guaiacum reaction remain negative. The oxidation of the R.F. is a reversible one, and the oxidised R.F. is reduced again by other oxidising systems. The function of this factor is thus that of a catalytic hydrogen carrier between the peroxidase and other oxidising or reducing systems. The R.F. is characterised by its reducing power.

*Part II.* It is shown that the adrenal cortex contains a relatively large quantity of a highly reducing substance which is specific for the interrenal system. The isolation of the substance is described. Chemical and physical properties of the substance are discussed. It is shown that the substance is a



Fig. 1. Cross-section of the adrenal gland after treatment with  $\text{AgNO}_3$ .

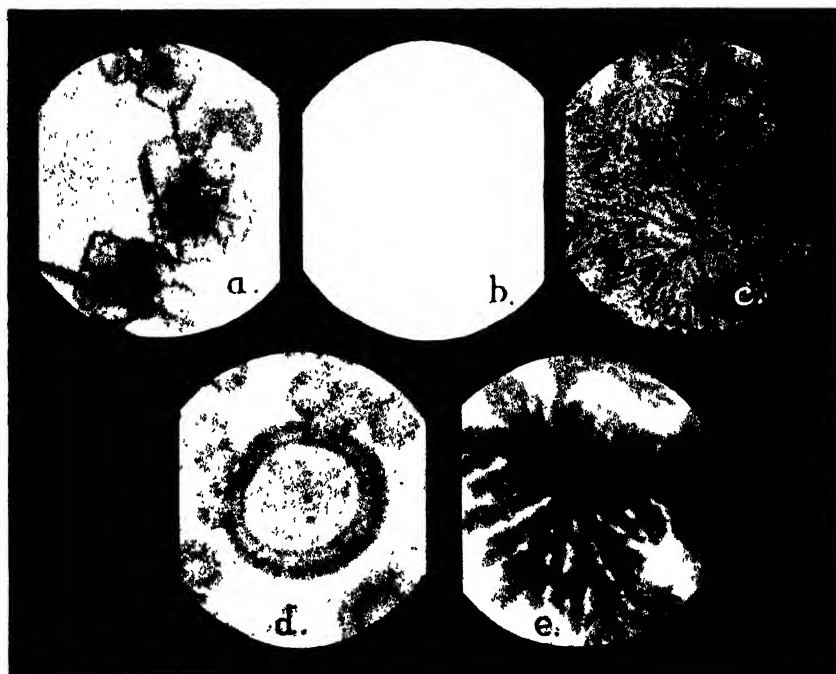


Fig. 2. Crystals of the hexuronic acid formed under different conditions.  
Diameter of each field 1.25 mm.



hitherto unknown, highly reactive isomer of glycuronic acid, so that the substance is a hexuronic acid.

*Part III.* The isolation of the hexuronic acid from oranges and cabbages is described. It is shown that this substance forms an essential part of the reducing factor, the chemistry of which is discussed.

*Part IV.* It is shown that the hexuronic acid has a definite reducing potential. On oxidation by mild oxidising agents the substance loses two atoms of hydrogen. This oxidation is reversible. The substance is autoxidisable. Molecular oxygen oxidises the substance irreversibly forming new carboxyl groups. This autoxidation is greatly catalysed by copper and inhibited by cyanide. Iron and manganese are relatively inactive as catalysts.

The substance is not oxidised immediately by peroxide and peroxidase. The peroxidase reaction is catalysed in the plant juice by phenols.

It is shown that in presence of peroxidase and a phenol the  $H_2O_2$  generated by other oxidising systems can be used for the oxidation of the hexuronic acid. Pigment formation in experiments *in vitro* can be inhibited by concentrations of 1 : 0.000025 of the acid.

In the absence of phenolic substances the hexuronic acid is not oxidised by the indophenoloxidase of animal tissues.

Haematin compounds (cytochrome) are reduced relatively slowly by the hexuronic acid.

The oxidised hexuronic acid is intensely reduced by animal tissues. The dehydrogenases of the type of the Schardinger enzyme and the succino-dehydrase are unable to reduce the oxidised hexuronic acid. The oxidised substance is reduced by the Hopkins glutathione system. Glutathione and fixed —SH strongly reduce the oxidised acid.

It is a great pleasure to express my deepest gratitude to Sir F. G. Hopkins for his extreme kindness and helpfulness in the course of this research.

#### REFERENCES.

- Battelli and Stern (1912). *Biochem. Z.* **46**, 317.  
Bertrand (1904). *Ann. Chim. Phys.* **8** (3), 187.  
Bezssonoff (1921). *Compt. Rend. Acad. Sci.* **173**, 466.  
Boutron (1886). *Compt. Rend. Acad. Sci.* **102**, 924.  
Ehrlich and Sommerfeld (1925). *Biochem. Z.* **168**, 263.  
Fleisch (1924). *Biochem. J.* **18**, 294.  
Keilin (1929). In the Press.  
Kilian (1922). *Ber. deutsch. chem. Ges.* **55**, 2817.  
Onslow (1921). *Biochem. J.* **15**, 113.  
Ruff (1899). *Ber. deutsch. chem. Ges.* **32**, 2269.  
Szent-Györgyi (1924, 1). *Biochem. Z.* **146**, 245.  
— (1924, 2). *Biochem. Z.* **150**, 195.  
— (1925, 1). *Biochem. Z.* **157**, 50, 68.  
— (1925, 2). *Biochem. Z.* **162**, 399.  
— (1927, 1). *Biochem. Z.* **181**, 425.  
— (1927, 2). *Biochem. Z.* **181**, 433.  
— (1927, 3). *Nature*, **119**, 782.  
Thurlow (1925). *Biochem. J.* **19**, 175.  
Wieland and Sutter (1928). *Ber. deutsch. chem. Ges.* **61**, 1060.  
Zilva (1927). *Biochem. J.* **21**, 689.  
— (1928). *Biochem. J.* **22**, 779.

## CLXXIV. NOTE ON THE REDUCTION OF THE DISULPHIDE GROUP BY ENZYME SYSTEMS.

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*(Received October 22nd, 1928.)*

At the end of a paper by Wieland and Bergel [1924] it was claimed that dithiodiglycollic acid was reduced to thioglycollic acid by succinic acid with the succinoxidase of washed liver mince.

Hopkins and Dixon [1922] had found that the succinoxidase system was quite unable to reduce oxidised glutathione. There were two points of difference between their experiments and those of Wieland and Bergel.

(a) Wieland and Bergel used dithiodiglycollic acid, whereas Hopkins and Dixon used glutathione.

(b) Wieland and Bergel used washed liver mince, whereas Hopkins and Dixon used washed muscle.

The experiments here described were begun with the object of determining which of these differences was responsible for the discrepancy in the results, but it was found that Wieland and Bergel's results could not be confirmed. Repeating their method with liver mince, and also using active enzyme extracts prepared from liver according to Ohlsson's method [1920], no increased reduction either of dithiodiglycollic acid or of glutathione was obtained in the presence of succinate.

Prof. Wieland has since kindly written to say that the work has been repeated but not confirmed in his laboratory.

It seemed possible that the disulphide-containing molecule might in some way become activated at a surface in the tissue, and in this state be liable to reduction not only by the tissue itself but by active hydrogen from other sources. As, however, enzyme systems producing active hydrogen are heterogeneous, the active hydrogen may not be available at the surface activating the disulphide which would also be a heterogeneous system. Methylene blue might, however, act as a homogeneous intermediary between the donator and acceptor. This possibility has been tested.

Xanthine oxidase and hypoxanthine were used as the primary source of active hydrogen, which kept the methylene blue reduced to the leuco-form, the experiment being carried out anaerobically. Muscle residue and oxidised glutathione were present, but again no more —SH was formed than in a control without hypoxanthine. Neither leucomethylene blue, therefore, nor hypoxanthine with xanthine oxidase, will reduce —SS— even in the presence of muscle residue.

Thus though attempts by several workers have been made to link up the disulphide group with hydrogen transportase systems, the thermostable tissue substance remains the only biological mechanism proved to reduce the disulphide group.

#### EXPERIMENTAL.

##### *Dithiodiglycollic acid with succinoxidase preparations and succinate.*

*Exp. 1.* Into a flask (*A*) 50 g. of liver mince thoroughly washed with distilled water were introduced, then 20 cc. of 2 % dithiodiglycollic acid solution at  $p_H$  8 in phosphate buffer, and 0.5 g. sodium succinate in 10 cc. buffer, and a few drops of chloroform. A control (*B*) containing exactly the same materials without succinate was also set up. The two flasks were filled with oxygen-free nitrogen and shaken for 7 hours at 37°. Then each was filtered and titrated with 0.1 *N* iodine in an atmosphere of nitrogen.

6.2 cc. of filtrate from (*A*) required 0.8 cc. 0.1 *N* iodine,

11.0 cc. of filtrate from (*B*) required 1.5 cc. 0.1 *N* iodine,

which is equivalent to 0.8 cc. iodine for 6.2 cc. of the liquid.

The above procedure follows the method of Wieland and Bergel, except that they make no mention of the  $p_H$  of the liquids, and less succinate and dithiodiglycollic acid were used than in their experiments. Also, instead of marking the filter flask so as to measure 10 cc. liquid for titration and finally squeezing out the tissue to estimate the total volume, the liquid filtered from each flask was measured after titration, and this volume and titre for the two flasks were compared.

*Exp. 2.* A similar experiment at the same initial  $p_H$  was carried out using water instead of buffer solution as medium.

24 cc. of filtrate (*A*) required 3.0 cc. 0.1 *N* iodine solution,

24.5 cc. of filtrate (*B*) required 3.1 cc. 0.1 *N* iodine solution.

*Exp. 3.* With the succinoxidase preparation, the flasks contained 25 cc. of the active alkaline phosphate extract from sheep's liver, 10 cc. of 2 % dithiodiglycollic acid in *M*/15 sodium phosphate brought to  $p_H$  8 with *N* NaOH solution, and in (*A*) 0.5 g. of sodium succinate. The two flasks were filled with oxygen-free nitrogen and shaken gently for 7 hours at 37°. Before filtering, 10 cc. of 10 % trichloroacetic acid were introduced, which by precipitating proteins gave easy filtration and a clear filtrate in which the —SH is not easily autoxidisable.

30.9 cc. filtrate (*A*) required 1.1 cc. 0.1 *N* iodine solution,

30.3 cc. filtrate (*B*) required 1.1 cc. 0.1 *N* iodine solution.

Thus, in no experiment has any extra reduction of —SS— occurred in the presence of succinate.

A similar experiment with glutathione and enzyme extract also showed no extra —SH formed in the presence of succinate.



*Glutathione and muscle residue with leucomethylene blue.*

*Exp.* 4. Two flasks were set up each containing 3 g. of thermostable residue, prepared according to Hopkins and Dixon's [1922] standard method from rabbit muscle, 25 cc. of a 10 % solution in phosphate buffer of xanthine oxidase-caseinogen preparation, prepared according to Dixon and Thurlow [1924], 10 cc. of a buffer solution containing 5 mg. oxidised glutathione per cc., and 2.5 cc. of 0.02 % methylene blue solution. The  $p_H$  of each was adjusted to 7.5, and 2.5 cc. of a solution containing 2 mg. hypoxanthine per cc. were added to (A), 2.5 cc. of water being added to (B).

The two flasks were filled with oxygen-free nitrogen and shaken for 7 hours at 37°. Then 15 cc. of 20 % trichloroacetic acid solution were run into each, the liquid was filtered off and titrated with 0.01 *N* iodine solution using the nitroprusside external indicator.

35 cc. of the liquid from both (A) and (B) required about 7 cc. of 0.01 *N* iodine.

Thus no extra —SH had been formed by the hypoxanthine in flask A donating hydrogen to the —SS— in presence of the muscle system, even with methylene blue as intermediary.

The enzyme preparations used in all cases were shown to be active by the reduction of methylene blue in the presence of their respective substrates.

## SUMMARY.

1. Neither dithiodiglycollic acid nor glutathione is reduced by succinoxidase and succinic acid.
2. No extra reduction of —SS— in presence of muscle residue is caused by the sources of active hydrogen tried.

## REFERENCES.

- Dixon and Thurlow (1924). *Biochem. J.* **18**, 971.  
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.  
Ohlsson (1920). *Skand. Arch. Physiol.* **41**, 78.  
Wieland and Bergel (1924). *Liebig's Ann.* **439**, 2, 209.

# CLXXV. THE SUPPOSED EFFECT OF TUMOUR EXTRACTS ON GLYCOLYSIS<sup>1</sup>.

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*(Received October 23rd, 1928.)*

It has been stated by Waterman [1924, 1925], that tumour extracts accelerate the glycolysis of rat and mouse kidney. Such an effect on glycolysis of normal tissues would be of great significance, and we have therefore undertaken experiments to test this finding.

Waterman gives little experimental detail in his earlier paper; a manometric method was used. In this author's later work [1925] the experimental conditions and methods of measuring glycolysis were very different from those of Warburg [1924].

Employing Warburg's methods, of which we have already made use in another connection [1928], we have been unable to find any effect of tumour extracts on glycolysis.

## EXPERIMENTS WITH TUMOUR EXTRACTS.

The tumour extracts were prepared in a similar manner to those of Waterman [1925]. Fresh, healthy tumour tissue was sliced, minced with scissors, and rubbed with sand and 0.16 *M* sodium chloride to form a suspension. After standing in the ice-chest the suspension was centrifuged, and the turbid supernatant fluid taken as the extract. Details of the extracts used are given in Table I. They are more concentrated than those of Waterman, who used 0.05–0.1 g. of tumour per cc. of saline, and extracted for 2–12 hours.

Table I. *Tumour extracts.*

Exp. No.	Tumour	g. tumour per cc. saline	Extraction time in hours
1	Flexner rat carc.	0.075	3½
2	" "	0.2	2
3	" "	0.3	3
4	" "	0.15	20
5	Human carc. groin	0.3	18
6	Rat sarcoma	0.14	2½
7	"	0.2	3½

In some of the experiments, the tissues of which the glycolysis was studied were used in the form of thin slices, as recommended by Warburg, in other

<sup>1</sup> This investigation was undertaken on behalf of the Liverpool Medical Research Organisation: Director, Prof. W. Blair Bell, of the University of Liverpool.

experiments the tissues were further cut up with scissors (as was done by Waterman). Where minced kidney was used, about 150 mg. of the tissue was suspended in 3 cc. of Ringer's solution in each manometric vessel, and to one or more of the vessels 0.3 cc. of the tumour extract was added. The same ratio of extract to Ringer's solution was kept in experiments where the tissue was in the form of slices, of which the wet weight varied from about 20 to 50 mg.

In measuring the effect of the extract on the glycolysis of tissue slices the technique was that described in our earlier paper. When minced tissue was employed the extract was usually added to some of the minces before measurements began. The glycolysis of the extract alone in Ringer's solution was also measured: this was small, but just measurable when the extraction time was brief (cells and cell fragments were present), and was subtracted from the glycolysis of the system (tissue + extract).

In Table II the percentage effects of the tumour extracts on the rate of anaerobic glycolysis of several rat tissues are given.

Table II. *Effect of tumour extracts on rate of anaerobic glycolysis of rat tissues.*

Exp. No.	Tissue	Form	Percentage effect of extract
1	Testis	Slices	- 2
2	Kidney	"	+ 1
3	"	"	- 4
4	Spleen	"	0
5	Kidney	Minced	- 2
6	"	Slices	+ 10
7	"	Minced	- 1
8	Spleen	Slices	+ 6
9	Kidney	"	- 5

The glycolysis of kidney tends to be irregular, both in amount and in rate of decrease with time. In all cases the effect of the extract on glycolysis lies within the experimental error.

#### EFFECT OF WASHING THE TISSUES ON THEIR SUBSEQUENT GLYCOLYSIS.

It was stated by Waterman [1925] that, if minced kidney tissue be washed twice with Ringer's solution followed by centrifuging, its glycolytic power disappears completely.

We have repeated the experiment. Waterman used 100 mg. of tissue and 2 cc. of a Ringer's solution. We washed 180 mg. of minced rat kidney twice with 4 cc. of Ringer's solution, and found that the rate of anaerobic glycolysis shown by the washed tissue was 93 % of that shown by the same weight of unwashed tissue. The difference from 100 % is within the experimental error.

Any other result would have been surprising, since, when working with Warburg's technique, a tissue slice which has been shaken in Ringer's solution for some time may be transferred to fresh Ringer's solution without appreciable effect on its glycolysis.

## DISCUSSION OF METHODS.

Whilst we are unable to offer any individual explanations to account for the effects observed by Waterman but not observed by us, we venture to think that this author's experimental conditions were unsatisfactory.

Waterman incubated 3 cc. of a Ringer-kidney mince, with or without 0.3 cc. of a tumour extract, for 2 hours in a 50 cc. conical flask containing air. The aerobiosis may have been incomplete and glycolysis may have taken place to some extent, as this author supposed.

We have made one or two experiments under somewhat similar conditions. To about 150 mg. of minced kidney were added 3 cc. of a Ringer's solution, with a concentration of bicarbonate similar to that used throughout by Waterman (0.0027 *M*, instead of the 0.02 *M* used by us unless otherwise stated), and previously saturated with nitrogen containing 5 %  $\text{CO}_2$ . The vessel containing the mince was bound to a manometer and an atmosphere of nitrogen (containing no  $\text{CO}_2$ ) introduced. After having been shaken for 10 minutes in the thermostat the manometers were read, and allowed to remain stationary for nearly 2 hours; they were then shaken for 10 minutes before being read again.

In both these and Waterman's experiments the  $p_{\text{H}}$  is ill-defined, and the bicarbonate concentration too low for full glycolytic power to be manifested. In addition, with such a large weight of tissue and such a low bicarbonate concentration, the system will show retention of carbon dioxide owing to the buffering power of the tissue, as is the case for serum [Warburg, 1925]. Both the manometric and acid-titration methods will on this account give results which are too low under Waterman's conditions.

The values derived by us for the glycolysis under these conditions ( $Q_{\text{CO}_2}^{\text{N}} = 0.2\text{--}0.3$ ) were about ten times smaller than those derived by Waterman under his not dissimilar conditions with the use of his acid-titration method. The  $p_{\text{H}}$  in Waterman's experiments may have been higher than in ours, but nevertheless the large difference in the results suggests that Waterman's titration method is in error. It appears, in fact, doubtful to what extent this method was properly tested and controlled. Thus, for instance, Ringer's solution seems to have been used as a "no glycolysis" control for a system containing tissue. It must be pointed out, however, that the bicarbonate content of the tissue should not be neglected when the Ringer's solution has a very low bicarbonate content.

Several experiments under the usual conditions of Warburg's technique, except in regard to the treatment of the tissue, showed that minced kidney usually glycolyses only at 30–50 % of the rate of sliced kidney,  $Q_{\text{CO}_2}^{\text{N}}$  having values of about 2 instead of 4–6. This is probably due to mechanical injury of the tissue in mincing. Respiration appears to behave in a similar way [Minami, 1923]. Furthermore, the rate of glycolysis of minced kidney decreases more rapidly with time than does that of sliced tissue.

Thus we have reason to believe that Waterman studied the behaviour of tissues which were damaged, and under conditions much more remote from the physiological than those of Warburg.

When to these two facts is added the use of an inaccurate, and perhaps insufficiently controlled, method of measuring glycolysis, it seems unlikely that results so obtained have any application to physiological conditions. It should perhaps be mentioned that we [1928] have been unable to confirm Waterman's finding [1925, 1926] that calcium salts affect glycolysis, nor could Krebs and Kubowitz [1927], working in Warburg's laboratory, confirm Waterman's statements [1926] that various sera affect glycolysis.

#### SUMMARY.

Saline extracts of malignant tumours have no effect on the anaerobic glycolysis of normal rat tissues. Washing tissues with a suitable Ringer's solution does not affect their glycolysis. Experimental conditions are discussed.

#### ADDENDUM.

(*Note added November 10th, 1928.*)

Kraut and Bumm have recently claimed [1928] that extracts of tumours and other tissues accelerate greatly the rate of glycolysis of kidney tissue. In the only detailed experiment given (Table I) it is evident, however, that these authors, following Warburg's [1923] earliest and only approximate technique for measuring anaerobic glycolysis, have misinterpreted their manometric changes. Glycolysis is erroneously taken as given by the difference between manometric changes shown by tissue slices in Ringer's solution (a) with, and (b) without, glucose. Furthermore, in calculating this difference no allowance appears to have been made for the different weights of the tissue slices used.

No increase in glycolysis due to the tissue extract is present, although claimed, in the experiment discussed, in which moreover the manometric changes recorded seem so small that the results cannot be accurate.

#### REFERENCES.

- Jowett and Brooks (1928). *Biochem. J.* **22**, 720.  
Kraut and Bumm (1928). *Z. physiol. Chem.* **177**, 125.  
Krebs and Kubowitz (1927). *Biochem. Z.* **189**, 194.  
Minami (1923). *Biochem. Z.* **142**, 334.  
Warburg (1923). *Biochem. Z.* **142**, 317.  
— (1924). *Biochem. Z.* **152**, 51.  
— (1925). *Biochem. Z.* **164**, 481.  
Waterman (1924). *Arch. Néerl. Physiol.* **9**, 573.  
— (1925). *Brit. J. Exp. Path.* **6**, 300.  
— (1926). *Ergeb. Inn. Med. Kinderheilk.* **30**, 304.

# CLXXVI. THE AMINO-ACID METHIONINE; CONSTITUTION AND SYNTHESIS.

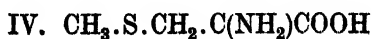
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(Received October 25th, 1928.)

As a result of certain bacteriological investigations, Mueller [1923, 1] isolated from caseinogen a new amino-acid,  $C_5H_{11}O_2NS$ , in a yield of 0.2–0.4 %. About the same quantity was obtained from egg-albumin, and smaller amounts are also present in edestin, wool and gelatin. That the substance is a primary cleavage product of protein, and not foreign to the body, was also indicated by its oxidation after administration to an animal, when the sulphur appears in the urine as inorganic sulphate [Mueller, 1923, 2]. The same amino-acid was subsequently found by Otake [1925]. On working up the residues of a commercial vitamin preparation from 6 tons of yeast, the latter obtained 0.6 g. of the pure amino-acid and published some useful photomicrographs of this substance and its derivatives.

The attention of one of us was called to this subject, in the course of an attempt, in conjunction with Dr A. S. Nayar, to isolate a thiolhistidine as precursor of ergothioneine, afterwards identified in red blood-corpuscles by Newton, Benedict and Dakin [1927] and by Eagles and Johnson [1927]. Whilst we could not obtain thiolhistidine, either from ergot or gelatin, we became involved in an examination of the non-cystine sulphur and prepared Mueller's amino-acid, following his directions. Mueller had already shown that the substance is not ethylcysteine. Since the methylthiol, like the methoxyl, group, is much more common in nature, we applied to the acid Kirpal and Bühn's method [1914] for the determination of  $CH_3S$ -groups and found one to be present. This result restricted the possible formulae to derivatives of butyric and isobutyric acids. Since all known cleavage products of protein are  $\alpha$ -amino-acids, only four formulae remained, viz.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -methylthiolbutyric acids (I–III) and methylthiolisobutyric acid (IV).



Of these (III) seemed much the most probable on account of its relation to cheirolin, a substance isolated from wallflower seeds (*Cheiranthus cheiri*) by Schneider [1910] and shown by him to have the constitution



Cheirolin would thus be the sulphone of a mustard oil, derived from Mueller's amino-acid in the same way as, for instance, phenylethyl mustard oil,  $\text{C}_6\text{H}_5.\text{CH}_2.\text{CH}_2.\text{N}:\text{C}:\text{S}$ , from the root of *Reseda* [Bertram and Walbaum, 1894], is derived from phenylalanine, via phenylethylamine. We therefore decided to synthesise the racemic substance (III) and indeed found it to be identical with Mueller's acid, except as regards optical activity [cf. Barger and Coyne, 1928]. Later, Dr Mueller informed us that he had himself arrived at the same constitution and had unsuccessfully attempted to prove it by synthesis. Since the amino-acid has a good title to be regarded as a constituent of protein, a shorter name than  $\gamma$ -methylthiol- $\alpha$ -aminobutyric acid seems desirable, and, after consultation with Dr Mueller, we suggest for it the name *methionine*, in allusion to the characteristic grouping.

Before we proceeded to synthesis, we attempted other means of breaking down the amino-acid, by decarboxylation, by oxidation and by exhaustive methylation, but, using only minute amounts of this rather inaccessible substance, we did not arrive at anything definite in this way. The synthesis was first attempted by various modifications of Erlenmeyer's method, from methylthiolacetaldehyde,  $\text{CH}_3.\text{S}.\text{CH}_2.\text{CHO}$ , which was easily made from bromoacetal. The condensation of the new aldehyde with hippuric acid and with diketopiperazine failed completely, but after prolonged experimentation we obtained a few grams of its condensation product with hydantoin in a state of purity, only to find that we were quite unable to reduce it to methionine; sulphur was eliminated. Thinking we could introduce the methylthiol group last, we attempted the condensation of phenoxyacetaldehyde,  $\text{C}_6\text{H}_5\text{O}.\text{CH}_2.\text{CHO}$ , with hydantoin, but without success.

We then had recourse to the old Strecker method [1850], from the next higher aldehyde,  $\text{CH}_3\text{S}.\text{CH}_2.\text{CH}_2.\text{CHO}$ , using a useful modification by Zelinsky and Stadnikoff [1908], and in this way obtained without special difficulty enough synthetic amino-acid to establish its identity with methionine. Whether from caseinogen or by synthesis, methionine is at present a very troublesome substance to prepare. We have tried to improve Mueller's method of isolation, but so far without any real success. Mercuric salts remain so far the only reagents which differentiate methionine somewhat from amino-acids like leucine and phenylalanine. Methionine ethyl ester is unstable when distilled, even in a charcoal vacuum, and a really characteristic derivative, from which methionine might be regenerated, is much to be desired.

## EXPERIMENTAL.

*Preparation of methionine.*

900 g. of gelatin, hydrolysed with hydrochloric acid and freed from the latter as far as possible by evaporation *in vacuo*, were dissolved in 2 litres of water and showed maximum precipitation on adding  $1\frac{1}{2}$  volumes of Hopkins's reagent (10 %  $\text{HgSO}_4$  in 5 %  $\text{H}_2\text{SO}_4$ ). The precipitate, after decomposition with  $\text{H}_2\text{S}$ , was esterified, the fraction boiling at  $130\text{--}150^\circ/0.1$  mm. was richest in sulphur, but most of the sulphur remained in the flask (as diketopiperazine?). Only a little impure methionine was obtained from the distillate.

By using Mueller's method, unmodified, we obtained 1–3 g. per kg. from caseinogen, with only a single precipitation with  $\text{HgCl}_2$ . The purity, as indicated by the sulphur content, was 85–95 %. A second precipitation with  $\text{HgCl}_2$  is very wasteful and was omitted for most purposes.

Mueller's method was examined in greater detail as follows. 2700 g. caseinogen were hydrolysed with sulphuric acid and the filtrate, neutralised with  $\text{NaOH}$ , was divided into halves. To one half were added 1350 g. of  $\text{HgSO}_4$  in 7 litres of 7 %  $\text{H}_2\text{SO}_4$ . The precipitate (*A*) was filtered off after 24 hours. The filtrate was half neutralised, causing a precipitate (*B*), 4–5 times as bulky as (*A*); neutralisation to Congo red of its filtrate yielded a still larger precipitate (*C*). The second half of the hydrolysate was treated with only one-third of the reagent added to this first half; the resulting precipitate (*D*) was filtered off, an additional third of the reagent was added, giving precipitate (*E*), and to its filtrate the third instalment of the reagent, giving (*F*). All six precipitates were washed twice by decantation, and twice by filtration, and extracted five times, with one litre of 2 % barium hydroxide each time. The five combined extracts were freed from barium hydroxide by sulphuric acid, from mercury by hydrogen sulphide, and the filtrate and washings were evaporated to 500 cc. A boiling saturated solution of 20–50 g. of mercuric chloride (according to the bulk of the original mercuric sulphate precipitate) was added. The mercuric chloride precipitate was freed from mercury by hydrogen sulphide, the filtrate was evaporated *in vacuo*, dissolved in the minimum quantity of boiling water and mixed with 5–10 cc. of pyridine (to remove  $\text{HCl}$ ) and three volumes of boiling alcohol. The following yields were obtained: *A*, 2 g. histidine with traces of methionine; *B*, 0.05 g. methionine; *C* was lost by accident; *D*, *E* and *F* respectively, 0.5, 0.5 and 0.6 g. methionine.

It would seem therefore that the larger quantity of the reagent should be added at once, and that the immediate precipitate, as well as that formed by half neutralisation, may be discarded.

*Determination of methylthiol grouping* according to Kirpal and Böhn [1914]. The cadmium sulphate bubbler was coloured yellow; hence some sulphur was split off as hydrogen sulphide. The methionine had a sulphur content of 18.9 % and was therefore 88 % pure.



**Analysis.** 0.2237 g. (crude) = 0.1968 g. (pure) gave 0.2998 g. AgI.

0.1023 g. (crude) = 0.0900 g. (pure) required 5.97 cc. *N*/10 AgNO<sub>3</sub>.

Found: CH<sub>3</sub>S = 30.5, 31.2; calc. for C<sub>5</sub>H<sub>11</sub>O<sub>2</sub>NS: CH<sub>3</sub>S = 31.6 %.

In the second estimation the methyl iodide was passed into pyridine, according to Kirpal and Bühn's second method [1915], and, after removal of the excess of pyridine, the methylpyridinium iodide was titrated. Ethyl iodide hardly reacts with pyridine in the cold. We moreover proved the identity of the alkyl iodide by converting the pyridinium salt into mercurichloride and comparing it with authentic specimens of methyl- and of ethyl-pyridinium mercurichloride.

	M.P.
A. Mercurichloride from methionine product ...	160–163°
B. Methylpyridinium mercurichloride ...	161–163°
C. Ethylpyridinium mercurichloride ...	131–133°
Mixture of A and B ...	160–163°
Mixture of A and C ...	110–119°

Hence the presence of one methylthiol grouping was established.

*Synthesis of methionine by Strecker's method.*

*β*-Methylthiolpropaldehydediethylacetal, CH<sub>3</sub>.S.CH<sub>2</sub>.CH<sub>2</sub>.CH(OC<sub>2</sub>H<sub>5</sub>)<sub>2</sub>, was prepared by passing excess of methyl mercaptan (2 mol.) into alcoholic sodium ethoxide solution and dropping (1 mol.) *β*-chloropropaldehydeacetal [Wohl, 1898] into it. B.P. 89°/14 mm., 96°/20 mm. Yield 70 % of the theoretical.

**Analysis.** 0.1124 g. gave 0.1456 g. BaSO<sub>4</sub>: S = 17.8 %.

0.1180 g. gave 0.1552 g. BaSO<sub>4</sub>: S = 18.0 %.

Calc. for C<sub>8</sub>H<sub>18</sub>O<sub>2</sub>S: S = 18.0 %.

For this and other syntheses the methyl mercaptan was prepared by hydrolysis of *S*-methylthiourea [Arndt, 1921]. The latter and the mercaptan were both obtained in a yield of 90 % of the theoretical. It is, however, most convenient to pass the mercaptan at once into sodium ethoxide solution, in a vessel provided with an ice-jacketed condenser, in order to keep back as much as possible of the malodorous mercaptan. Since the chloro- and the methylthiol-acetal have nearly the same boiling-point, a large excess of mercaptan is used in order to make the reaction go completely and avoid the necessity of a separation.

*β*-Methylthiolpropaldehyde, CH<sub>3</sub>.S.CH<sub>2</sub>.CH<sub>2</sub>.CHO. The acetal was hydrolysed by boiling for half an hour with two volumes of water containing 1–2 cc. of dilute hydrochloric acid. The aldehyde separated on cooling, and was extracted with ether. B.P. 60°/12 mm. Yield, 80 % of the theoretical.

*α*-Amino-*γ*-methylthiolbutyronitrile, CH<sub>3</sub>.S.CH<sub>2</sub>.CH<sub>2</sub>.CH(NH<sub>2</sub>)CN. To a well-cooled mixture of the aldehyde in ether and one equivalent of ammonium chloride in concentrated aqueous solution there was added slowly one equivalent of potassium cyanide in the minimum quantity of water, with frequent shaking. Excess of ammonium chloride (2–3 g.) was then added and the mixture was

shaken for 6 hours. The ethereal layer was separated and the aqueous layer was thoroughly extracted with ether. The total ether extract was dried over sodium sulphate and dry hydrogen chloride was passed into the well-cooled solution. A brown semi-crystalline syrup separated and the ether could be decanted off. Attempts to obtain the pure aminonitrile hydrochloride failed, so the crude product was used.

*$\alpha$ -Amino- $\gamma$ -methylthiolbutyric acid*,  $\text{CH}_3\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\text{COOH}$ . After pouring off the ether, two volumes of concentrated hydrochloric acid were added, and the solution was boiled for 2 hours, during which the original dark brown colour deepened and a tar separated. The hydrochloric acid was removed as far as possible by vacuum distillation and the semi-crystalline residue, containing a large amount of tar and ammonium chloride, was dissolved in the minimum amount of boiling water. 10 cc. of pyridine were added to remove free hydrochloric acid; on adding three volumes of boiling alcohol the amino-acid separated in clusters of narrow platelets. It was recrystallised from alcohol and melted at  $281^\circ$  with decomposition. The yield was on the average 6 % of the theoretical, calculating from the aldehyde used.

The last reaction is the one unsatisfactory feature of this synthesis. The following improvements were attempted.

(1) Washing the acid solution of the aminonitrile with ether, and boiling with hydrochloric acid, without separating the crude aminonitrile hydrochloride. In this way the large amount of inorganic salt remains in the solution, and the amino-acid has to be precipitated with mercuric chloride; only a trace of the amino-acid was obtained in this manner.

(2) Hydrolysis of the aminonitrile by dilute hydrochloric acid. This did not prevent formation of tar and gave much poorer results than concentrated hydrochloric acid.

(3) Hydrolysis by barium hydroxide. The tar separated on the walls of the flask and the solution remained clear. After 10 hours' boiling, the barium was removed quantitatively and the filtrate was evaporated. Only a little gum remained, which gave the ninhydrin reaction and contained sulphur, but could not be crystallised.

(4) The original condensation was repeated with five equivalents of ammonium chloride and potassium cyanide, but this gave much more tar and only a minute amount of the amino-acid.

(5) A slightly improved yield resulted when the ethereal solution of the aldehyde was added to a concentrated aqueous solution, containing one equivalent each of potassium cyanide and ammonium chloride. The solution was kept ice-cold and stirred vigorously.

#### *Analysis.*

5.91 mg. gave 0.551 mg. N (micro-Kjeldahl).

9.38 mg. gave 14.5 mg.  $\text{BaSO}_4$  (micro-Carius).

Found: 9.3 % N; 21.3 % S.

Calc. for  $\text{C}_5\text{H}_{11}\text{O}_2\text{NS}$ : 9.4 % N; 21.5 % S.

*α-Naphthylcarbamido-derivative*,  $\text{CH}_3.\text{S}.\text{CH}_2.\text{CH}_2.\text{CH}(\text{NH}.\text{CO}.\text{NH}.\text{C}_{10}\text{H}_7)\text{COOH}$ . 0.1 g. methionine, both synthetic and natural, in 6.8 cc. of *N*/10 sodium hydroxide was mixed with 0.12 g. *α*-naphthyl isocyanate and shaken for one hour, then filtered. The clear filtrate was just acidified with hydrochloric acid and a bulky precipitate was thrown down (excess of acid causes the precipitate to coagulate to a sticky mass which cannot be crystallised). The precipitate was filtered off, thoroughly washed and dried. It crystallised after dissolving in boiling alcohol and adding three volumes of boiling water. On cooling short stout needles separated. Some difficulty was experienced in obtaining good crystals and the product often separated in a semi-amorphous, gelatinous condition, even when an alcoholic solution was left to evaporate at room temperature. Mixtures of benzene with acetone and alcohol are unsuitable. Similar difficulties are not uncommon with naphthylcarbamido-derivatives.

In the case of natural methionine the *α*-naphthylcarbamido-derivative was obtained at the second attempt with the melting-point  $187^\circ$ , that given by Mueller. The derivative of synthetic methionine, however, was only obtained in imperfect crystals, in spite of several attempts, and melted at  $181\text{--}2^\circ$ . The mixture with the derivative of the natural substance also melted at this temperature. Some other derivatives were therefore made.

*Methionine thiohydantoin*,  $\text{CH}_3.\text{S}.\text{CH}_2.\text{CH}_2.\text{CH}.\text{CO}$ . Following Johnson



and Nicolet [1913], 0.1 g. methionine and 0.25 g. ammonium thiocyanate were added to 3 cc. of acetic anhydride containing two drops of glacial acetic acid. The mixture was heated on the water-bath for 5 minutes, when solution was complete. Further heating does not improve the yield. 10 cc. of water were added, when a reddish-brown oil separated. The mother liquor was decanted, and attempts were made to crystallise the oil. Since this failed, 2 cc. of concentrated hydrochloric acid were added and the mixture was evaporated to dryness. This removed acetyl groups and a crystalline residue was obtained. Extraction with hot acetone and concentration gave fine yellow needles. These were recrystallised from hot alcohol, when they became colourless. A small additional crop was obtained by evaporating the aqueous mother liquor to dryness with 3 cc. of concentrated hydrochloric acid and extracting the residue with hot acetone. The total yield was poor. It was later improved by extracting the oil with chloroform.

#### *Analysis.*

5.76 mg. gave 0.841 mg. N (micro-Kjeldahl).

8.53 mg. gave 21.06 mg.  $\text{BaSO}_4$  (micro-Carius).

Found: 14.6 % N; 33.8 % S.

Calc. for  $\text{C}_6\text{H}_{10}\text{ON}_2\text{S}_2$ : 14.7 % N; 33.7 % S.

The thiohydantoin of the natural amino-acid, that of the synthetic acid, and the mixture of both, all melted at  $146^{\circ}$ , proving their identity.

*Methionine picrolonate.* 0.1 g. of the amino-acid and 0.18 g. picrolonic acid were dissolved in 5 cc. of water; the solution was boiled for 10 minutes and then evaporated on the steam-bath. The residual sticky syrup was dried *in vacuo* over sulphuric acid and then extracted with hot acetone. When thoroughly dried the picrolonate is not very soluble in cold acetone and crystallises from fairly dilute solution in sickle-shaped pale yellow crystals. It is extremely soluble in water and alcohol, and gives a syrup on evaporation of its solution in either solvent.

The picrolonate of natural methionine melted at  $178^{\circ}$ , that of synthetic at  $178^{\circ}$ , and their mixture at  $177$ – $178^{\circ}$ . This also proves the identity. A slight darkening occurs at  $173^{\circ}$  together with softening, but the actual melting and decomposition point is sharply defined at  $178^{\circ}$ .

*Attempted synthesis by Erlenmeyer's method.*

This is only of interest in so far as it illustrates the limitations of a favourite way of synthesising amino-acids, which has of late been very useful in connection with thyroxine, 3 : 4-dihydroxyphenylalanine, and other aromatic amino-acids [cf. Harington and McCartney, 1927]. Erlenmeyer [1893, 1899, 1901, 1904] synthesised only two aliphatic amino-acids, leucine and serine, and described in the former case the condensation product of *isobutyraldehyde* and hippuric acid as an oil "welches nach längerem Stehen zu krystallisieren beginnt." The difficulty with aliphatic aldehydes seems to be that the condensation product is so soluble that it does not readily crystallise out and thus inhibits the reaction. The success with aromatic aldehydes can hardly depend on the acidic character of the benzene nucleus, for Pyman made histidine by this method. In the case of methionine hippuric acid and diketopiperazine were quite useless and the preparation of a few grams of the condensation product with hydantoin required much labour.

*Methylthiolacetal*,  $\text{CH}_3\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH}(\text{OC}_2\text{H}_5)_2$ . This was the first compound of the kind we made. Methyl mercaptan [Arndt, 1921], from 50 g. of *S*-methylthiourea sulphate and 70 cc. of 5 *N* sodium hydroxide, was passed into a solution of 7 g. of sodium in alcohol and then 30 g. of bromoacetal [Fischer and Landsteiner, 1892] were dropped in. The reaction flask and condenser were cooled by ice. Sodium bromide separated at once; next day it was filtered off and after addition of water the acetal was extracted with ether. B.P.  $188$ – $190^{\circ}/760$  mm. Yield, 80 % of the theoretical.

*Analysis.*

0.1628 g. gave 0.2307 g.  $\text{BaSO}_4$ .

0.1405 g. gave 0.2045 g.  $\text{BaSO}_4$ .

Found: S = 19.9 %.

Calc. for  $\text{C}_7\text{H}_{16}\text{O}_2\text{S}$ : S = 19.5 %.

*Methylthiolacetaldehyde*,  $\text{CH}_3\cdot\text{S}\cdot\text{CH}_2\cdot\text{CHO}$ , was prepared by boiling the acetal for half an hour with 1 %  $\text{HCl}$ , and extraction with ether. The crude aldehyde boiled at  $120\text{--}140^\circ$ ; yield, 65 %.

*Methylthioethylidene hydantoin*,  $\text{CH}_3\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH} : \text{C} \begin{array}{l} \text{NH}\cdot\text{CO} \\ | \\ \text{CO}\cdot\text{NH} \end{array}$ . Using the

ordinary method of condensation with sodium acetate and acetic anhydride, we at first obtained only a very soluble brown oil. Later, following Wheeler and Hoffman [1911], we used glacial acetic acid instead of the anhydride (which causes acetylation) and two equivalents of sodium acetate. After boiling for 2 hours under reflux, water was added, a little tar was removed by ether, the solution was neutralised and extracted with chloroform. The latter on evaporation left a brown syrup which partly crystallised; the crystals were dried on a plate, washed with ether, recrystallised from acetone and then from water. m.p.  $156^\circ$ . Yield, 50 mg. from 2 g. aldehyde or 1 % of the theoretical. Potassium acetate (a better dehydrating agent) increased the yield to 2–3 %. Condensation at  $150\text{--}200^\circ$  in a sealed tube was useless. Ultimately we got a yield of nearly 20 % with acetic acid and potassium acetate, by improving the method of extraction. After boiling for 2 hours the acetic acid was removed by vacuum distillation and the semi-solid residue extracted with chloroform. The extract was washed with sodium carbonate and water, dried, and evaporated. It was dissolved in a little chloroform and ether was cautiously added. This caused some of the condensation product to crystallise. The mother liquor was evaporated, the residue was dissolved in acetone and again treated with ether, giving a further yield of crystals, a maximum total of 1 g. from 3 g. aldehyde.

*Analysis.*

7.25 mg. gave 1.178 mg. N (micro-Kjeldahl).

7.50 mg. gave 1.216 mg. N (micro-Kjeldahl).

0.0690 g. gave 0.0934 g.  $\text{BaSO}_4$ .

0.1030 g. gave 0.1403 g.  $\text{BaSO}_4$ .

Found: N, 16.3, 16.2 %; S, 18.6, 18.7 %.

Calc. for  $\text{C}_6\text{H}_8\text{O}_2\text{N}_2\text{S}$ : N, 16.3 %; S, 18.6 %.

*Reduction of the hydantoin condensation product.* Sodium amalgam in cold absolute alcohol produced no change. Sodium in hot alcohol gave a few crystals, m.p.  $210\text{--}213^\circ$ , but mostly an uncrystallisable syrup; judging by the odour, a volatile sulphur compound was given off during the reduction. Zinc dust and 75 % acetic acid gave the same odour, a syrup and some unchanged substance. Aluminium amalgam gave a similar result.

*Attempted condensation of hydantoin and phenoxyacetaldehyde.* The acetal of the latter was prepared according to Pomeranz [1894] and more conveniently by dissolving one part of sodium in three equivalents of phenol, adding one equivalent of bromoacetal, boiling for 4 hours and then steam-distilling from alkaline solution; yield, 70 %. The free aldehyde, on condensation with hydantoin by various methods, gave much tar and no crystals.

*$\beta$ -Methylthiolpropionic acid*,  $\text{CH}_3\text{S}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$ , was prepared from mercaptan and ethyl- $\beta$ -iodopropionate. The methylthiol ester distilled at  $192^\circ/760$  mm.,  $95^\circ/20$  mm.; yield, 60 % of the theoretical. It was hydrolysed by boiling for 4 hours with *N* hydrochloric acid. The acid was a colourless oil, B.P.  $235\text{--}240^\circ/760$  mm.

*Analysis.*

0.0961 g. gave 0.1857 g.  $\text{BaSO}_4$ .

0.1863 g. neutralised 15.60 cc. 0.1 *N* NaOH.

S = 26.6 %. Equivalent = 119.4.

Calc. for  $\text{C}_4\text{H}_8\text{O}_2\text{S}$ : S = 26.7 %. Equivalent = 120.

*$\beta$ -Methylsulphonepropionic acid*,  $\text{CH}_3\cdot\text{SO}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{COOH}$ , was obtained by oxidising 1.7 g. of the methylthiol acid, accurately neutralised with potassium hydroxide, at  $0^\circ$  with 1.5 g. of potassium permanganate in 100 cc. of water. The sulphone acid was extracted, after acidification and evaporation, by means of alcohol and crystallised at  $105^\circ$  as stated by Schneider [1910], who obtained it by oxidation of the amine from cheirolin. This sulphone acid is very stable and boils without decomposition below  $200^\circ$  at 4 mm. We synthesised this acid and its precursor with a view to obtaining them as degradation products of methionine, before the latter was synthesised. For the characterisation of methionine a sulphone may become useful, but, since the oxidation is irreversible, a sulphone derivative would be useless for isolating methionine. A sulphoxide would be preferable.

We desire to express our gratitude to Dr J. Howard Mueller of Boston for his sympathetic attitude to our work, which he assisted in the later stages by a supply of methionine. We also gratefully acknowledge a grant from the Moray Fund of Edinburgh University.

REFERENCES.

- Arndt (1921). *Ber. deutsch. chem. Ges.* **54**, 2236.  
 Barger and Coyne (1928). *Arch. Sci. Biol.* **12**, 141.  
 Bertram and Walbaum (1894). *J. prakt. Chem.* **50**, 555.  
 Eagles and Johnson (1927). *J. Amer. Chem. Soc.* **49**, 575.  
 Erlenmeyer, jun. (1893). *Liebig's Ann.* **275**, 1.  
 — (1899). *Liebig's Ann.* **307**, 138.  
 — (1901). *Liebig's Ann.* **316**, 145.  
 — (1904). *Liebig's Ann.* **337**, 205.  
 Fischer and Landsteiner (1892). *Ber. deutsch. chem. Ges.* **25**, 2551.  
 Harington and McCartney (1927). *Biochem. J.* **21**, 854.  
 Johnson and Nicolet (1913). *Amer. Chem. J.* **9**, 197.  
 Kirpal and Böhn (1914). *Ber. deutsch. chem. Ges.* **47**, 1084.  
 — (1915). *Monatsh.* **36**, 853.  
 Mueller (1923, 1). *J. Biol. Chem.* **56**, 156.  
 — (1923, 2). *J. Biol. Chem.* **58**, 373.  
 Newton, Benedict and Dakin (1927). *J. Biol. Chem.* **72**, 367.  
 Otake (1925). *Biochem. Z.* **161**, 446.  
 Pomeranz (1894). *Monatsh.* **15**, 379.  
 Schneider (1910). *Liebig's Ann.* **375**, 207.  
 Strecker (1850). *Liebig's Ann.* **75**, 29.  
 Wheeler and Hoffman (1911). *Amer. Chem. J.* **45**, 368.  
 Wohl (1898). *Ber. deutsch. chem. Ges.* **31**, 1796, cf. (1900) **33**, 276.  
 Zelinsky and Stadnikoff (1908). *Ber. deutsch. chem. Ges.* **41**, 2061.

## CLXXVII. THE BIOLOGICAL INERTNESS OF IRRADIATED MYCOSTEROLS OTHER THAN ERGOSTEROL.

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*(Received October 29th, 1928.)*

THE evidence so far available indicates that of the three groups of "protoplasmic" sterols, the zoo-, phyto- and myco-sterols, only one member of the last group, *i.e.* ergosterol, can be antirachitically activated by irradiation. Besides ergosterol there is present in ergot a second mycosterol, to which Tanret [1908] has given the name fungisterol. In view of the apparently unique property of ergosterol, it became essential to study the behaviour of fungisterol in this direction. Our previous attempt to settle this question gave indecisive results [Rosenheim and Webster, 1927], partly owing to the difficulty of separating the substance from crude ergosterol, containing only about 10 % fungisterol, but mainly owing to the small total amount of sterols obtainable from ergot. We have since been able, through the kindness of Mr F. H. Carr, of the British Drug Houses, Ltd., to work up the mother-liquors resulting from the recrystallisation of large amounts of ergosterol from ergot. The biological examination of the sterol isolated by us led to the conclusion that the slight antirachitic activity acquired by it on irradiation was due to contamination with traces of ergosterol. This result is in agreement with the spectroscopic analysis which indicated the presence of less than 5 % of ergosterol in this specimen of fungisterol. Another specimen of an apparently identical sterol, prepared from ergot, was kindly put at our disposal by Messrs Burroughs, Wellcome and Co. and gave identical results. The physical constants of the two sterols agree with each other, but not with those of fungisterol, as described by Tanret. Apparently the latter product was still a mixture of several sterols, of which two further constituents were isolated and are being investigated chemically in Messrs Burroughs, Wellcome and Co.'s laboratories. Their biological examination, which we carried out, also gave negative results.

These facts lend further confirmation to the view expressed previously [Rosenheim and Webster, 1928] that ergosterol is the only substance which can be converted into vitamin D by irradiation.

## EXPERIMENTAL.

*Chemical.* The residue of the mother-liquors after removal of crystallisable ergosterol, representing the yield from about 250 kg. ergot, was a yellowish powder (5.5 g.) which melted at 107–110° and had  $[\alpha]_{5461}^{19^\circ} - 63^\circ$ . The product was acetylated and the acetate recrystallised from acetic anhydride. The apparently uniform product (M.P. 117–118°,  $[\alpha]_{5461}^{19^\circ} - 50^\circ$ ) was fractionated from methyl alcohol at various temperatures. The fractions obtained at 36° and 18°, containing presumably the bulk of the less soluble ergosterol, were discarded, and the colourless crystals deposited at – 5° from the filtrates were again recrystallised from methyl alcohol. The final product weighed 0.195 g.,  $[\alpha]_{5461}^{20^\circ} - 43^\circ$  (*c* in chloroform = 1), M.P. 117–118°, solidified at 102° (a mixed M.P. with cholesterol acetate, 107–108°).

The sterol isolated in Messrs Burroughs, Wellcome and Co.'s experimental laboratories had a constant rotation of  $[\alpha]_{5461} - 34^\circ$  (*c* in chloroform = 1.5). It had no definite melting point, but the acetate melted at 117–120°. A mixed M.P. of this and our own preparation showed no depression. The two specimens appear to be identical, but both were still contaminated with traces of ergosterol, as shown by a specific colour reaction (to be published shortly) and by their spectroscopic examination.

Tanret's fungisterol had M.P. 144° and  $[\alpha]_D - 22^\circ$ ; the acetate M.P. 158.5° and  $[\alpha]_D - 15.9^\circ$ . There is no criterion by which Tanret's success in freeing his product from ergosterol may be judged, and, although its physical constants differ from those of the presumably purer preparations above described, it seems advisable to retain the name "fungisterol" for the sterol which constitutes the largest amount in the sterol mixture accompanying ergosterol in ergot. It is interesting to note that zymosterol which occurs together with ergosterol in yeast [Smedley-MacLean, 1928] is evidently not identical with fungisterol.

The following information was kindly supplied to us by Messrs Burroughs, Wellcome and Co. with regard to the two other sterols isolated by them from ergot.

Sterol (1) had  $[\alpha]_{5461} + 60^\circ$  (*c* in chloroform = 0.9). It melted at 172° and gave a benzoate M.P. 173° (mixed M.P. of sterol with its benzoate 150°) and  $[\alpha]_{5461} + 72^\circ$  (*c* in chloroform = 0.8).

Sterol (2). A substance giving the colour reaction of a sterol. It had M.P. ca. 260°,  $[\alpha]_{5461} - 90^\circ$  (*c* in alcohol = 0.2).

*Spectroscopic examination.* An alcoholic solution of fungisterol (1 : 20,000) showed no absorption when compared on the same plate with a solution of ergosterol of the same strength, in which the characteristic bands were conspicuous. An approximate match with the latter was obtained only when the strength of the fungisterol solution was increased to 1 : 1000. The ergosterol content of this specimen was therefore probably less than 5 %, and that of the second specimen was found to be of the same order.



Sterol (1) showed no absorption even in the highest strength tested (1 : 1000), and gave an entirely negative test with the above-mentioned specific colour reaction for ergosterol. It may therefore be considered as practically free from ergosterol.

Sterol (2). Spectroscopic examination indicated about 8 % ergosterol as impurity. The colour reaction for ergosterol was strongly positive.

*Biological examination.* This was carried out by our usual technique. The sterols were irradiated in alcoholic solution and the modified Sherman-Pappenheimer diet was used in the feeding experiments. Continued experience with this technique in a large number of tests on hundreds of rats has shown us that the minimum effective dose of irradiated ergosterol lies between 1/10,000 and 1/20,000 mg. *per diem*. On the assumption that the ergosterol percentage of the specimens of fungisterol, as shown by spectroscopic analysis, was of the order of 5 %, the doses were so adjusted that the minimum dose of ergosterol, which would be effective after irradiation, was present in the largest dose (1/500 mg.) of fungisterol, whilst the smallest dose (1/5000 mg.) of fungisterol, although still double the amount of the effective dose of ergosterol, would only contain about 1/100,000 mg. of the latter. The results were decisive and in general agreement with the spectroscopic test, showing that doses of 1/5000 and 1/2500 mg. of irradiated fungisterol did not prevent severe rickets, whilst the largest dose induced healing, though not as complete as in the control experiments receiving 1/20,000 mg. of irradiated ergosterol. This result justifies the conclusion that the effect produced by these specimens of irradiated fungisterol is due to the ergosterol still contained in them as impurity. A similar series of experiments was found to be conclusive in demonstrating the biological inactivity of irradiated zymosterol, one of the mycosterols of yeast [Hume, Smith and Smedley-MacLean, 1928].

Sterol (2) gave practically identical results, whilst sterol (1) proved to be entirely inactive, even in the largest doses administered, thus again confirming the evidence of the spectroscope and the colour reaction.

#### SUMMARY.

A mycosterol, accompanying ergosterol in ergot, was isolated, for which Tanret's name "fungisterol" is retained. This, as well as two other sterols from the same source, was found to be biologically inactive after irradiation. The results confirm the evidence previously adduced for the unique function of ergosterol as the mother-substance of vitamin D.

#### REFERENCES.

- Hume, Smith and Smedley-MacLean (1928). *Biochem. J.* **22**, 980.  
Rosenheim and Webster (1927). *Lancet*, ii, 621.  
——— (1928). *Biochem. J.* **22**, 762.  
Smedley-MacLean (1928). *Biochem. J.* **22**, 22.  
Tanret (1908). *Ann. Chim. Phys.* **15**, 313.

## CLXXVIII. RESOLUTION OF *dl*-THYROXINE.

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*(Received October 31st, 1928.)*

OWING to the alkaline hydrolysis which, so far as we know at present, is an essential step in any method for the isolation of thyroxine from the thyroid gland, the fact that the compound is always obtained in the racemic condition is not surprising. It is, however, to be assumed that, in the natural state, thyroxine occurs as one or other optically active modification, and, further, that the physiological activity of the naturally occurring isomeride may be considerably greater than that of its enantiomorph.

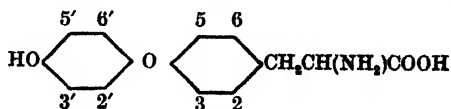
The fact that so-called natural thyroxine, as we know it, is the *dl*-compound rendered the complete chemical and physiological identification of the natural and synthetic substance an easy matter. Should the naturally occurring variety of thyroxine be considerably more active physiologically than its isomeride, an explanation of the discrepancy observed by certain workers [cf. Reid Hunt, 1922] between the physiological activity of thyroxine and of amounts of thyroid gland containing equivalent quantities of iodine, would be afforded by the fact that the thyroxine had been artificially racemised, for in the process of racemisation the thyroxine might lose anything up to one-half of its physiological activity depending on the relationship between the activities of the two isomerides.

The best solution of the problem would obviously be the isolation of optically active thyroxine from the thyroid gland. A large number of experiments in this direction have been made, but so far all have proved fruitless. As is indicated by the experiments of earlier workers on the subject and as fully borne out by the experience of the present writer, acid hydrolysis is entirely useless. From the constitution of thyroxine indeed this is to be expected; since 3 : 5-diiodotyrosine, in which the iodine atoms are present in a configuration similar to that of the 3' : 5'-iodine atoms of thyroxine, is also readily destroyed by boiling with acids. Assuming the necessity of a drastic hydrolysis then, a necessity which is indicated by all the available evidence, the only possible line of attack seems to be through the action of enzymes. Many attempts in this direction have been made, but success has not as yet attended any of them. One of the main difficulties in this connection is the fact observed many years ago by Oswald [1909], and again by the

present writer [Harington, 1926], that certain preparations of trypsin have the property of splitting off more or less of the iodine of the thyroid gland as iodide. Certain experiments have indeed indicated that this is not a universal property of all trypsin preparations, and the hope has not been abandoned that a preparation may be obtained which will effect the desired hydrolysis; it is evident, however, that many further experiments will be required in order to determine the necessary conditions.

Since no further immediate progress could be made in this direction, it was decided to tackle the question from the other end, and to attempt the resolution of synthetic *dl*-thyroxine. It will be evident from the description which has already been given of the properties of thyroxine that the chief difficulty to be anticipated in this work is insolubility, which makes the preparation and purification of salts a troublesome task. In order to minimise this difficulty it was decided, in the first instance, to resolve *dl*-3 : 5-diiodothyronine<sup>1</sup> into its optically active components, and then to iodinate these to the corresponding optically active thyroxine. *dl*-3 : 5-Diiodothyronine, on warming with anhydrous formic acid, readily yielded a formyl derivative, a substance readily soluble in alcohol but quite insoluble in water. It was first attempted to prepare salts of this compound with alkaloids such as brucine, strychnine, or cinchonine which have been used with success in the resolution of the formyl derivatives of other *dl*-amino-acids. The alkaloid salts of *dl*-formyldiiodothyronine, however, were compounds with unattractive properties. They were almost entirely insoluble in water, but little soluble in absolute alcohol, and fairly readily soluble in mixtures of the two solvents. They invariably separated from solution as oils which could in no case be induced to crystallise; in short, it became evident that they were useless for the purpose in view. It appeared from this experience that one had to seek not only for greater solubility but for greater tendency to crystallise; that is to say, that the employment of a base with lower molecular weight was more likely to give favourable results. With this in view attention was directed to the employment of  $\alpha$ -phenylethylamine, and the use of this base has led to at least a partial success. On warming equivalent amounts of the acid and base in a large volume of water, solution took place, and on cooling about 70 % of the acid separated in the form of the phenylethylamine salt. This insoluble fraction could not be obtained optically pure by recrystallisation; the mother liquor on the other hand yielded a soluble fraction which after one or two recrystallisations appeared pure; the acid was recovered from this salt, the

<sup>1</sup> In order to lessen the clumsiness of the systematic nomenclature of thyroxine derivatives it is proposed to call the amino-acid, desiodothyroxine, "thyronine," the positions being numbered as shown



so that thyroxine would be "3 : 5 : 3' : 5'-tetraiodothyronine."

formyl group removed by hydrolysis with hydrobromic acid, and the resulting optically active diiodothyronine iodinated in the ordinary way. Since the insoluble fraction could not be purified it was necessary to employ in turn the two isomerides of the base; in this way, using *l*- $\alpha$ -phenylethylamine there was obtained *l*-thyroxine having  $[\alpha]_{5461}^{21^\circ} - 3.2^\circ$  and, using *d*- $\alpha$ -phenylethylamine, *d*-thyroxine having  $[\alpha]_{5461}^{21^\circ} + 2.97^\circ$ . I am indebted to Dr J. H. Gaddum for the physiological investigation of these samples of optically active thyroxine. A brief account of his results is included in this paper, and the work will be more fully described elsewhere. Briefly, according to his results, *l*-thyroxine is about three times as active physiologically as the *d*-compound. If we assume that the pure *d*-compound is inactive, this result would indicate that each isomeride was contaminated with about 25 % of the other; the chemical work seems to indicate that the separation was probably better than this, but the point must remain in doubt until natural optically active thyroxine has been obtained. At present the most that can be said is that *l*-thyroxine is very definitely the more active physiologically, and is therefore presumably the naturally occurring isomeride.

The numerical value of the rotation of optically active thyroxine according to these experiments is indeed surprisingly low, and it might be that partial racemisation had been induced during the final iodination of the diiodothyronine. In order to control this point, an experiment was performed in which *l*-tyrosine was iodinated and the resulting 3 : 5-diiodotyrosine reduced to tyrosine again by shaking in alkaline solution with hydrogen and palladium-calcium carbonate. The tyrosine finally obtained in this way showed the same optical rotation as the starting material. If the analogy holds, therefore, as seems not unreasonable to expect, it should be possible to assume that no racemisation has accompanied the introduction of the last two iodine atoms into thyroxine.

#### EXPERIMENTAL.

*Formyl-3 : 5-diiodothyronine.* 15 g. *dl*-3 : 5-diiodothyronine<sup>1</sup> were warmed on the water-bath for 3 hours with 100 cc. of 99 % formic acid; the solution was evaporated under diminished pressure and the residual syrup warmed for a further 3 hours with 50 cc. of formic acid; the process was once more repeated, distillation of the formic acid this time leaving a crystalline residue; the latter was extracted with warm absolute alcohol; a small amount of unchanged amino-acid remained undissolved and was removed by filtration; the filtered solution was boiled with charcoal, filtered and poured into a large volume of hot water; on slow cooling the formyl derivative crystallised out in colourless plates, m.p. 207°; the yield was 12 g. The compound was practically insoluble in water; it was readily soluble in alcohol, but sparingly so in other organic solvents.

<sup>1</sup> For the 3 : 5-diiodothyronine used in this work and in that described in the following paper I am indebted to Messrs Hoffmann la Roche of Basel to whom, and in particular to Dr M. Guggenheim, I wish to express my best thanks.

*Analysis.* 1.13 mg. required 4.9 cc. *N*/200 thiosulphate [Kendall, 1914].  
22.8 mg. gave 0.537 mg. N (micro-Kjeldahl).

	I	N
Calculated for $C_{18}H_{13}O_5NI_2$	45.9	2.5
Found ... ..	46.0	2.4

*dl*- $\alpha$ -Phenylethylamine, prepared by the reduction of acetophenoneoxime with sodium and alcohol, was resolved by the method of Aeschlimann [1925]: the *l*-base had  $[\alpha]_{5461}^{20^\circ} - 41.0^\circ$  and the *d*-base  $[\alpha]_{5461}^{10^\circ} + 40.5^\circ$  in benzene solution.

A preliminary experiment was carried out as follows: 11.06 g. *dl*-formyl-3 : 5-diiodothyronine were suspended in 1100 cc. of boiling water and treated with 2.42 g. *l*- $\alpha$ -phenylethylamine dissolved in 200 cc. of warm water; after boiling for a minute the acid had passed into solution; a trace of impurity was removed by filtration and the solution allowed to cool slowly and stand for 24 hours at the ordinary temperature; the salt which separated was crystalline but evidently not homogeneous, appearing under the microscope as clumps of stout needles mixed with a felt of fine ones. It was filtered off, washed with water and dried; it amounted to 8.35 g. (theoretical 6.74 g.). On concentrating the mother liquor under diminished pressure to about 100 cc. the more soluble salt separated at first as an oil, which, however, soon crystallised to an apparently homogeneous felted mass of fine colourless needles (3.7 g.). The latter salt was fairly soluble in alcohol, whilst the first fraction seemed to be much less so; the 8.35 g. were therefore ground up with cold alcohol, filtered off and washed with alcohol; it was then recrystallised by dissolving in dilute alcohol and boiling until the alcohol was removed; it still, however, did not appear homogeneous. The two fractions were then decomposed and the optical rotations of the acids observed; that from the soluble salt had  $[\alpha]_{5461}^{23^\circ} + 21.3^\circ$ ; that from the insoluble salt  $[\alpha]_{5461}^{23^\circ} - 16.9^\circ$ .

In the light of the above results the following series of experiments were carried through; repetition of the resolution has given essentially similar results.

#### A. *l*-Thyroxine.

6.1 g. formyl-*dl*-3 : 5-diiodothyronine were converted into the salt with *l*-phenylethylamine under the conditions described above; the insoluble salt amounted to 4.6 g. and the soluble salt to 2.5 g.; the latter had  $[\alpha]_{5461}^{22^\circ} + 22.0^\circ$  (*c* = 5 in 50 % alcohol); after two recrystallisations from water it had  $[\alpha]_{5461}^{22^\circ} + 23.8^\circ$  under the same conditions; it crystallised in masses of fine colourless needles which were anhydrous and melted at 188–189°.

*Analysis.* 1.26 mg. required 4.45 cc. *N*/200 thiosulphate [Kendall, 1914].  
15.3 mg. gave 0.637 mg. N (micro-Kjeldahl).

	I	N
Calculated for $C_{24}H_{24}O_5N_2I_2$	37.7	4.2
Found ... ..	37.4	4.2

*Formyl-l-3 : 5-diiodothyronine.* The above salt was dissolved in dilute alcohol and decomposed by the addition of slightly more than the theoretical amount of dilute hydrochloric acid. Precipitation of the acid was completed by cautious dilution with water. The formyl-*l-3 : 5-diiodothyronine* formed colourless plates, darkening at 195° and melting at 214° (decomp.). In 5 % solution in alcohol in a 2 dm. tube  $\alpha = 2.78^\circ$ , whence  $[\alpha]_{5461}^{21^\circ} = +27.8^\circ$ .

*l-3 : 5-Diiodothyronine.* The formyl derivative was boiled for 1 hour under a reflux condenser with 15 % hydrobromic acid; the solution was evaporated to dryness under diminished pressure and the residual hydrobromide dissolved in warm aqueous alcohol; the solution was then cautiously neutralised with ammonia whereupon the amino-acid separated in glistening colourless plates, m.p. 256° (decomp.).

In 5.4 % solution in 0.880 ammonia it had  $[\alpha]_{5461}^{20^\circ} - 1.3^\circ$ .

*l-Thyroxine.* The above amino-acid was iodinated in ammoniacal solution as previously described [Harrington and Barger, 1927], and the thyroxine purified through the sodium salt. Mention may here be made of the observation that in this final step in the synthesis of thyroxine it is better to employ the strongest iodine solution (*i.e.* above 2.5 *N*); by adopting this measure the greater part of the thyroxine is obtained as the ammonium salt, and the yield is raised to 75–80 % of the theoretical. The *l*-thyroxine obtained in this experiment melted at 235–236° with decomposition; 0.66 g. was dissolved in 6.07 g. of 0.5 *N* sodium hydroxide and 13.03 g. of alcohol; under these conditions it had  $[\alpha]_{5461}^{21^\circ} - 3.2^\circ$ .

### B. *d*-Thyroxine.

The steps in the preparation of *d*-thyroxine were precisely similar, so that detailed description is not necessary.

11.06 g. formyl-*dl-3 : 5-diiodothyronine* were converted into the salts with *d*- $\alpha$ -phenylethylamine. The soluble fraction amounted to 3.6 g. and, on recrystallisation, formed colourless needles, melting at 187–188°, and having  $[\alpha]_{5461}^{19.5^\circ} - 21.9$  in 5 % solution in 50 % alcohol.

*Formyl-d-diiodothyronine.* On decomposition the above salt gave formyl-*d-3 : 5-diiodothyronine*, which formed colourless plates melting at 210°, and having, in 5 % solution in alcohol  $[\alpha]_{5461}^{21^\circ} - 26.9^\circ$ .

*d-3 : 5-Diiodothyronine.* The formyl derivative, on hydrolysis, yielded *d-3 : 5-diiodothyronine*, m.p. 256° with decomposition. In 4.35 % solution in 0.880 ammonia this had  $[\alpha]_{5461}^{18^\circ} + 1.15^\circ$ .

*d-Thyroxine.* The thyroxine prepared by iodination of the above amino-acid melted at 237° with decomposition, and had  $[\alpha]_{5461}^{21^\circ} + 2.97^\circ$  (0.74 g. dissolved in 6 g. of 0.5 *N* sodium hydroxide and 14 g. of alcohol).

*C. Effect of iodination on optical activity of tyrosine.*

The *l*-tyrosine used in these experiments had  $[\alpha]_{5461}^{23^\circ} - 12.0^\circ$  (4.8 % solution in *N* hydrochloric acid). For the preparation of 3 : 5-diiodotyrosine, the tyrosine was dissolved in 20 parts of concentrated ammonia (Sp. Gr. 0.880); the solution was cooled in ice and treated drop by drop with a concentrated (2.5 *N*) solution of iodine in potassium iodide until the theoretical 2 mols. had been added. On removal of the ammonia by distillation *in vacuo*, or by spontaneous evaporation, the diiodotyrosine crystallised out; it was filtered off, washed with cold water, and recrystallised from 50 % acetic acid with the addition of charcoal. The yield was 60 % of the theoretical. The product, in 4.8 % solution in *N* hydrochloric acid, had  $[\alpha]_{5461}^{23^\circ} + 2.6^\circ$ .

The diiodotyrosine so obtained was dissolved in 100 parts of *N* potassium hydroxide and the solution shaken in an atmosphere of hydrogen in presence of the palladium-calcium carbonate catalyst, exactly as described for the reduction of thyroxine [Harington, 1926]; the theoretical uptake of hydrogen was complete within 45 minutes. The catalyst was removed by filtration and the solution neutralised with acetic acid and concentrated on the water-bath; the yield of tyrosine was about 80 % of the theoretical, and the product had  $[\alpha]_{5461}^{23^\circ} - 11.8^\circ$  (4.8 % solution in *N* hydrochloric acid). No racemisation had therefore been induced by iodination followed by reduction.

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Samples of the optical isomers obtained from Dr Harington have been tested for their effects on the growth of tadpoles, and on the oxygen consumption and weight of rats.

*Effect on tadpoles.*

Evidence has been presented elsewhere [Gaddum, 1927] in support of the belief that, under suitable conditions, the effect of thyroxine on tadpoles may be used as a specific quantitative test for this substance. The optical isomers were compared by the method described in the above-mentioned paper. The tadpoles are immersed in batches of 12 for 24 hours in dilute solutions of the substance to be tested. They are then kept in tap water and the average length of each batch is determined at intervals. Several such tests were performed and it was consistently found that both the *d*-thyroxine and the *l*-thyroxine produced, in small concentrations, definite effects on the length and development and also that the *l*-thyroxine produced these effects in slightly smaller concentrations than the *d*-thyroxine. No definite value has yet been obtained by this method for the proportion between the potencies of the preparations.

*Effect on rats.*

The oxygen consumption was determined by a method devised by Richards and Collison [1928]. This method enables a continuous record both of the oxygen consumption and of the movements of the animal to be kept, and, for the determination of the basal oxygen consumption, periods have been chosen during which the record showed no movement. The rats were kept without food each night, and each morning they were weighed and their basal oxygen consumptions were measured. When these had reached a fairly steady level, the thyroxine was injected subcutaneously (2 mg. of thyroxine being dissolved in 1 cc. of *N*/100 NaOH). It was found that this injection produced a rise in oxygen consumption lasting 3–14 days. The experiments are not yet complete, but it is already clear that both preparations produce definite effects in doses of 4 mg. per kg. both on the weight and on the oxygen consumption. The *l*-thyroxine appears to be about three times as potent as the *d*-thyroxine.

*Conclusion.*

The two tests applied both point to the conclusion that the *l*-thyroxine is definitely more active physiologically than the *d*-thyroxine, but that the latter possesses considerable activity.

There are two possible explanations of this fact.

## REFERENCES.

- Aeschlimann (1925). *J. Chem. Soc.* **127**, 811.  
Gaddum (1927). *J. Physiol.* **64**, 246.  
Harington (1926). *Biochem. J.* **20**, 293.  
Harington and Barger (1927). *Biochem. J.* **21**, 169.  
Kendall (1914). *J. Biol. Chem.* **19**, 251.  
Oswald (1909). *Z. physiol. Chem.* **62**, 432.  
Reid Hunt (1922). *Amer. J. Physiol.* **63**, 257.  
Richards and Collison (1928). *J. Physiol.* **66**, 299.



## CLXXIX. SOME DERIVATIVES OF THYROXINE.

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SINCE thyroxine has been available in the pure condition its therapeutic use has been limited by the fact that it is only possible to elicit with certainty the full physiological activity of the substance when it is administered intravenously. When thyroxine or its sodium salt is administered by the mouth absorption appears to be irregular, and scarcely ever is the full effect of a given dose obtained. Except, therefore, in hospital practice where the intravenous injection offers no difficulties, thyroxine compares unfavourably with crude desiccated thyroid gland as a therapeutic agent. In our opinion thyroxine fails to be absorbed after oral administration owing to its extreme insolubility when in the free condition. The necessity of a drastic hydrolysis of the thyroid gland before thyroxine can be isolated points to the probability that the compound exists in the gland as a constituent of a peptide or protein, which one would expect to be readily soluble in water. If this hypothesis be correct one may assume that after ingestion of the whole gland substance, the active principle is either absorbed as a peptide or is liberated by enzymic action, but liberated in a physical condition favourable to absorption. It was, therefore, thought worth while to attempt the preparation of some simple peptides containing thyroxine, in order to see whether a relatively soluble compound could be obtained, and, if so, whether such a compound, when administered by mouth, would exercise the physiological effect of thyroxine with a certainty comparable to that attainable by the intravenous injection of the latter. The preparation of glycyl- and *dl*-alanyl-thyroxine was undertaken and is described in detail below. Unfortunately, however, the introduction of a single amino-acid residue is insufficient to affect the solubility of thyroxine to an appreciable extent; these two peptides are very nearly as insoluble in water as is thyroxine itself. On administration to rats by mouth they give similar effects to those obtained by the oral administration of equivalent amounts of the sodium salt of thyroxine, over which therefore they offer no therapeutic advantage. This result is however of some interest, in that it indicates either that these peptides must undergo hydrolysis in the body, in spite of their insolubility, or that the blocking of the amino-group of

thyroxine in this way offers no obstacle to the exercise of the typical physiological effect. As might be expected, the preparation of even the simple peptides containing thyroxine offers considerable technical difficulties owing to the extreme insolubility of all thyroxine derivatives; it was not therefore considered practicable to pursue this line of investigation further by attempting the preparation of more complex thyroxine-containing peptides.

It was thought that the desired increase in solubility might be attained by the acylation of thyroxine with a hydroxy-acid, and with this object *N*-lactylthyroxine has been synthesised. Here again, however, disappointment was encountered, in that the *N*-lactyl derivative, whilst being extremely soluble in alcohol, was almost insoluble in water. It must, therefore, be confessed that, up to the present, no practicable method of increasing the solubility of thyroxine has been devised.

It is convenient also to include in this paper an account of the amines corresponding to thyroxine and to 3 : 5-diiodothyronine; the latter amino-acid was readily decarboxylated by a modification of the method recently described by Johnson and Daschavsky [1925] and Abderhalden and Gebelein [1926] and the resulting amine could be iodinated to give thyroxamine. But little physiological interest appears to attach to these substances; when tested on tadpoles [Gaddum, 1927] thyroxamine shows similar effects to those obtained with thyroxine; neither of the amines, however, shows any marked pressor effect such as might have been anticipated from their constitution. Their preparation and properties are only recorded as a matter of chemical interest.

#### EXPERIMENTAL.

##### A. *Glycyl- and dl-alanyl-thyroxine.*

The preparation of these peptides was accomplished in two ways: (a) by coupling chloroacetyl chloride and  $\alpha$ -bromopropionyl chloride respectively with the methyl ester of 3 : 5-diiodothyronine, followed by hydrolysis of the ester group, treatment with ammonia, and finally introduction of the remaining two iodine atoms; (b) by coupling the acid chlorides with thyroxine methyl ester, followed by hydrolysis of the ester group and treatment with ammonia.

(a) *3 : 5-Diiodothyronine methyl ester hydrochloride.* The amino-acid was suspended in five parts of methyl alcohol and the solution saturated with a vigorous stream of dry hydrogen chloride; after cooling, the saturation was repeated. The solution was concentrated to a small volume by distillation under diminished pressure whereupon the ester hydrochloride crystallised out; it was filtered off and a small further crop was obtained by cautious addition of ether to the mother liquor. The whole was recrystallised from dilute alcohol containing some hydrochloric acid, and formed colourless needles, m.p. 230° (decomp.). The yield was 87 % of the theoretical. The compound was but little soluble in water or absolute alcohol, but fairly readily soluble in mixtures of the two solvents.

*Analysis.* 1.33 mg. required 5.5 cc. *N*/200 thiosulphate [Kendall, 1914].  
31.1 mg. gave 0.79 mg. N (micro-Kjeldahl).

		I	N
Found	... ..	43.8	2.5
Calculated for $C_{16}H_{16}O_4NClH_2$		44.1	2.4

*3 : 5-Diiodothyronine methyl ester.* The ester hydrochloride was dissolved in 50 % alcohol and the cold solution treated with one equivalent of 0.1 *N* sodium hydroxide; crystallisation rapidly set in and was completed by dilution with water. The yield was quantitative. On recrystallisation from dilute alcohol the ester formed clusters of small needles melting at 174–175°. It was insoluble in water, fairly soluble in alcohol, but very sparingly so in all other ordinary organic solvents.

*Analysis.* 2.07 mg. required 9.25 cc. *N*/200 thiosulphate [Kendall, 1914].  
30.1 mg. gave 0.76 mg. N (micro-Kjeldahl).

		I	N
Found	... ..	47.4	2.5
Calculated for $C_{16}H_{15}O_4NI_2$ ...		47.1	2.6

*Chloroacetyl-3 : 5-diiodothyronine methyl ester.* Owing to the great insolubility of the amino-acid ester in benzene and chloroform, the coupling with the acid chloride could not be conveniently brought about in these solvents; for the purposes of this reaction it was found better to employ anisole as a solvent; 0.54 g. of the ester was dissolved in 12 cc. of redistilled anisole and the solution cooled in ice; with continual cooling and good shaking 0.125 g. of chloroacetyl chloride was added; the solution was then shaken with 0.4 g. of sodium bicarbonate dissolved in a little water and a further addition of 0.125 g. of chloroacetyl chloride was made. The solution was filtered from a trace of undissolved matter and the anisole layer separated and dried over calcium chloride; the anisole was removed by distillation at a pressure of 1–2 mm., the bath temperature not being raised above 60°. The oily residue was dissolved in a little warm benzene; on spontaneous evaporation of the solvent the ester crystallised out and was filtered off and washed with ether. The yield was 75–80 % of the theoretical. Recrystallised from dilute alcohol it formed fine hair-like needles, M.P. 160°. It was readily soluble in alcohol and benzene.

*Analysis.* 1.72 mg. required 6.65 cc. of *N*/200 thiosulphate [Kendall, 1914].  
33.3 mg. gave 0.72 mg. N (micro-Kjeldahl).

		I	N
Found	... ..	41.2	2.3
Calculated for $C_{18}H_{16}ONClH_2$		41.0	2.2

*Chloroacetyl-3 : 5-diiodothyronine.* The above ester was dissolved in *N* sodium hydroxide (two equivalents) and the solution was allowed to stand for 15 minutes at room temperature; two equivalents of *N* hydrochloric acid were then added, whereupon the acid separated as an oil which, on rubbing, solidified; it was purified by dissolving in hot 30 % alcohol, from which, on slow cooling,

it separated as a sphaero-crystalline powder; on heating it sintered at 156° and melted at 166–168°.

*Analysis.* 1.66 mg. required 6.55 cc. *N*/200 thiosulphate [Kendall, 1914].  
35.7 mg. gave 0.851 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	41.8	2.4
Calculated for $C_{17}H_{14}O_5NClH_2$	42.2	2.3

This acid was easily soluble in alcohol and glacial acetic acid, but sparingly so in other organic solvents and in water.

*Glycyl-3 : 5-diiodothyronine.* Chloroacetyl-3 : 5-diiodothyronine was covered with a large excess of 25 % aqueous ammonia and allowed to stand in a sealed tube at the ordinary temperature for 5 days. The contents of the tube were then mixed with an equal volume of alcohol, and evaporated under diminished pressure. The residue was extracted with hot absolute alcohol, and the insoluble portion crystallised from 30 % alcohol from which it separated in colourless needles. On heating, it darkened above 150°, but did not melt definitely below 290°.

*Analysis.* 1.59 mg. required 6.5 cc. *N*/200 thiosulphate [Kendall, 1914].  
15.5 mg. gave 0.716 mg. N (micro-Kjeldahl).  
26.5 mg. gave 1.24 cc. moist N at 758 mm. and 21° (Van Slyke).

	I	N	NH <sub>2</sub> -N
Found ... ..	43.3	4.6	2.6
Calculated for $C_{17}H_{16}O_5N_2I_2$	43.6	4.8	2.4

The compound was almost insoluble in water and alcohol, and only very sparingly soluble in mixtures of these two solvents.

*Glycylthyroxine.* Glycyl-3 : 5-diiodothyronine was dissolved in concentrated ammonia and iodinated in the manner employed for the preparation of thyroxine; towards the end of the addition of the iodine, the product began to separate as a micro-crystalline powder. After standing in the cold for some time this material was separated on the centrifuge; it was purified by dissolving in strong ammonia, from which, on spontaneous evaporation of the latter, it separated as a micro-crystalline powder. For analysis it was dried *in vacuo* at 110° over phosphorus pentoxide.

*Analysis.* 1.13 mg. required 6.43 cc. *N*/200 thiosulphate [Kendall, 1914].  
17.6 mg. gave 0.595 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	60.3	3.4
Calculated for $C_{17}H_{14}O_5N_2I_4$	60.9	3.4

The preparation of *dl*-alanyl-3 : 5-diiodothyronine was carried out by a precisely similar series of reactions which do not call for any detailed comment.

*$\alpha$ -Bromopropionyl-3 : 5-diiodothyronine methyl ester.* This was prepared similarly to the corresponding chloroacetyl compound. On recrystallisation

from dilute alcohol, it formed clusters of colourless needles, melting at 161–162°.

*Analysis.* 1.78 mg. required 6.3 cc. *N*/200 thiosulphate [Kendall, 1914].  
38.1 mg. gave 0.751 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	37.5	1.9
Calculated for $C_{18}H_{18}O_5NBrI_2$	37.7	2.1

*$\alpha$ -Bromopropionyl-3 : 5-diiodothyronine.* The above ester was hydrolysed by treatment with *N* sodium hydroxide (two equivalents) at the ordinary temperature for 15 minutes. On acidification, the crude acid separated as an oil which slowly solidified; this was dissolved in dilute sodium carbonate, and the sodium salt of the acid precipitated by addition of saturated sodium chloride; the sodium salt was redissolved and again precipitated, and was then converted into the free acid, which was crystallised first from dilute acetic acid and then from dilute alcohol. It formed a sphaero-crystalline powder, melting at 194–195°. It was readily soluble in alcohol and glacial acetic acid, sparingly so in other organic solvents and in water.

*Analysis.* 2.05 mg. required 7.4 cc. *N*/200 thiosulphate [Kendall, 1914].  
42.3 mg. gave 0.876 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	38.3	2.1
Calculated for $C_{18}H_{18}O_5NBrI_2$	38.5	2.1

*dl-Alanyl-3 : 5-diiodothyronine.* This was prepared by the action of 25 % aqueous ammonia solution on the above acid for 5 days at the ordinary temperature. On slow separation from its solution in hot 30 % alcohol, it formed a sphaero-crystalline powder, m.p. 207°.

*Analysis.* 1.83 mg. required 7.29 cc. *N*/200 thiosulphate [Kendall, 1914].  
20.1 mg. gave 0.905 mg. N (micro-Kjeldahl).  
30.4 mg. gave 1.2 cc. moist N at 763 mm. and 21° (Van Slyke).

	I	N	NH <sub>3</sub> -N
Found ... ..	42.2	4.5	2.2
Calculated for $C_{18}H_{14}O_5N_2I_2$	42.6	4.7	2.3

The solubilities of this dipeptide were similar to those of the corresponding glycyl derivative, but its conversion into alanylthyroxine by iodination did not proceed satisfactorily, the product being extremely difficult to purify. The preparation of alanylthyroxine, as also a second synthesis of glycylthyroxine, was therefore carried out, using thyroxine itself as a starting point, by the following series of reactions.

(b) *Thyroxine methyl ester hydrochloride.* Thyroxine (5.8 g.) was covered with methyl alcohol (60 cc.) and the solution saturated with a vigorous stream of dry hydrogen chloride; after cooling, the saturation was repeated, and the solution was then concentrated by distillation under diminished pressure. The crystalline precipitate was filtered off, washed with alcohol and ether, and

dried. The yield was 90 % of the theoretical. For analysis it was recrystallised from dilute alcohol containing a little hydrochloric acid, when it formed colourless needles melting at 221.5° with decomposition. The ester hydrochloride was sparingly soluble in water or alcohol, fairly readily so in mixtures of the two solvents.

*Analysis.* 1.03 mg. required 5.93 cc. *N*/200 thiosulphate [Kendall, 1914].  
33.6 mg. gave 0.536 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	61.0	1.6
Calculated for $C_{16}H_{14}O_4NClH_4$	61.4	1.7

*Thyroxine methyl ester.* The ester hydrochloride was dissolved in 50 % alcohol and the solution treated with 2*N* sodium hydroxide (one equivalent). Crystallisation of the ester commenced immediately, and was completed by addition of water and standing in the cold for some time. Recrystallised from dilute alcohol, it formed small prismatic needles melting at 156°. The ester was readily soluble in alcohol, but sparingly so in all the usual organic solvents and in water.

*Analysis.* 1.04 mg. required 6.18 cc. *N*/200 thiosulphate [Kendall, 1914].  
41.8 mg. gave 0.697 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	63.0	1.7
Calculated for $C_{16}H_{13}O_4NI_4$	63.4	1.7

*Chloroacetylthyroxine methyl ester.* Thyroxine methyl ester (0.8 g.) was dissolved in redistilled anisole (16 cc.); the solution was cooled in ice and treated gradually, with continued cooling and good shaking, with 0.12 g. of chloroacetyl chloride dissolved in 4 cc. of anisole; 0.4 g. of sodium bicarbonate in a little water was added, and a further addition of 0.12 g. of chloroacetyl chloride was made. The anisole layer was separated and dried over calcium chloride, and the anisole removed by distillation under diminished pressure; the oily residue was rubbed up with light petroleum, whereupon it solidified. It was recrystallised from benzene, from which it separated in colourless prisms, melting at 159–160°; the yield was 80 % of the theoretical. The compound was easily soluble in alcohol and benzene, sparingly so in light petroleum and insoluble in water.

*Analysis.* 1.54 mg. required 8.4 cc. *N*/200 thiosulphate [Kendall, 1914].  
31.5 mg. gave 0.478 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	58.9	1.5
Calculated for $C_{18}H_{14}O_5NClH_4$	58.6	1.6

*Chloroacetylthyroxine.* The above ester was dissolved in 50 % alcoholic *N* sodium hydroxide (two equivalents) and the solution was allowed to stand at the ordinary temperature for 20 minutes; at the end of this period hydrochloric acid (2 equivalents) was added; the precipitated acid was filtered off

and dried, the crude yield being practically quantitative. On slow separation from its solution in dilute acetic acid, the compound formed a colourless sphaero-crystalline powder, which melted with decomposition at 201–202°. It was readily soluble in alcohol and glacial acetic acid, but practically insoluble in other organic solvents and in water.

*Analysis.* 1.58 mg. required 8.65 cc. *N*/196 thiosulphate [Kendall, 1914].  
30.1 mg. gave 0.468 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	59.1	1.55
Calculated for $C_{17}H_{12}O_5NClH_4$	59.5	1.6

*Glycylthyroxine.* Chloroacetylthyroxine (0.3 g.) was heated with 25 % aqueous ammonia (10 cc.) in a sealed tube at 100° for 1 hour; after cooling, the solution was filtered from a trace of insoluble matter and allowed to evaporate spontaneously, whereupon the peptide separated as a micro-crystalline powder, contaminated with ammonium chloride. When the solution was reduced to a small bulk the solid was filtered off and dried; it was then extracted with boiling absolute alcohol, and the insoluble residue was dissolved in alcohol with the addition of a little concentrated aqueous ammonia; on long standing the peptide separated as a crystalline powder, which was filtered off, washed with alcohol and ether, and dried at 110° *in vacuo* over phosphorus pentoxide. It melted with decomposition at 188–190°, and was identical with the product described above as resulting from the iodination of glycyl-3 : 5-diiodothyronine.

*Analysis.* 1.16 mg. required 6.6 cc. *N*/200 thiosulphate [Kendall, 1914].  
15.2 mg. gave 0.536 mg. N (micro-Kjeldahl).  
16.4 mg. gave 0.51 cc. moist N at 180–752 mm. (Van Slyke).

	I	N	NH <sub>2</sub> -N
Found ... ..	60.3	3.5	1.8
Calculated for $C_{17}H_{14}O_5N_2I_4$	60.9	3.4	1.7

*α-Bromopropionylthyroxine methyl ester.* This was prepared similarly to the chloroacetyl compound by the coupling of *α*-bromopropionyl chloride with thyroxine methyl ester in anisole solution. The yield was 70 % of the theoretical. The compound was crystallised from benzene and formed masses of prismatic needles which melted with decomposition at 199–201°.

*Analysis.* 1.55 mg. required 7.86 cc. *N*/196 thiosulphate [Kendall, 1914].  
31.9 mg. gave 0.495 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	54.7	1.6
Calculated for $C_{19}H_{16}O_5NBrI_4$	54.9	1.5

*α-Bromopropionylthyroxine.* The above ester was hydrolysed with cold sodium hydroxide in the usual manner, and the crude acid purified by recrystallisation from dilute acetic acid, from which it separated first as an oil which

subsequently solidified to a felted mass of fine needles, melting at 193–194° with decomposition.

*Analysis.* 1.11 mg. required 5.8 cc. *N*/200 thiosulphate [Kendall, 1914].  
45.2 mg. gave 0.663 mg. N (micro-Kjeldahl).

	I	N
Found ... ..	55.4	1.5
Calculated for $C_{18}H_{14}O_5NBrI_4$	55.7	1.5

*Alanylthyroxine.*  $\alpha$ -Bromopropionylthyroxine (0.25 g.) was heated with 25 % aqueous ammonia (9 cc.) in a sealed tube at 100° for 1 hour; the reaction mixture was worked up as described for glycylthyroxine. The separation of the alanyl compound was considerably slower, and the yield was poorer (25 % of the theoretical). The compound, which formed a colourless powder with no definite crystalline structure, melted with decomposition at 195–200° after sintering at 185°. In physical properties it was similar to glycylthyroxine. For analysis it was dried *in vacuo* at 110° over phosphorus pentoxide.

*Analysis.* 1.24 mg. required 6.98 cc. *N*/200 thiosulphate [Kendall, 1914].  
15.3 mg. gave 0.497 mg. N (micro-Kjeldahl).  
15.4 mg. gave 0.45 cc. moist N at 18°/752 mm. (Van Slyke).

	I	N	NH <sub>3</sub> -N
Found ... ..	59.6	3.2	1.7
Calculated for $C_{18}H_{16}O_5N_2I_4$	59.9	3.3	1.65

#### B. *N*-Lactylthyroxine.

*N*-Acetylactylthyroxine methyl ester. Acetylactyl chloride was prepared by the method of Anschütz and Bertram [1904]. Thyroxine methyl ester (1.6 g.) was dissolved in redistilled anisole (32 cc.); to the solution, cooled in ice, was gradually added a solution of acetylactyl chloride (0.5 mol.) dissolved in anisole. The precipitate of thyroxine methyl ester hydrochloride was filtered off and amounted to 0.8 g. The anisole was removed from the filtrate by distillation *in vacuo*, and the residual oil dissolved in benzene in which it was very soluble; addition of light petroleum precipitated the ester, the crude yield being quantitative. It was purified by recrystallisation from light petroleum.

*Analysis.* 1.43 mg. required 7.46 cc. *N*/196 thiosulphate [Kendall, 1914].

	I
Found ... ..	56.3
Calculated for $C_{21}H_{19}O_7NI_4$	56.1

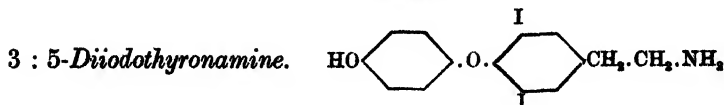
*N*-Lactylthyroxine. The above ester was dissolved in *N* sodium hydroxide (3 mols.), and the solution was allowed to stand for 20 minutes at the ordinary temperature; a slight excess of dilute hydrochloric acid was then added; the precipitated acid was filtered off, washed with water, and recrystallised first from dilute alcohol and then from dilute acetic acid. It formed bunches of colourless needles melting at 199–200° with decomposition. It was very readily soluble in alcohol and glacial acetic acid, but almost insoluble in water.



*Analysis.* 1.19 mg. required 6.58 cc. *N*/196 thiosulphate [Kendall, 1914].

			I
Found	...	...	59.6
Calculated for	$C_{18}H_{15}O_6NI_4$		59.8

C. *Amines.*



When 3 : 5-diiodothyronine was heated with diphenylamine according to the method described by Abderhalden and Gebelein [1926], decarboxylation took place, but there was considerable formation of tar and the yield of amine was poor; the operation was more successful when air was excluded from the reaction flask. Finely powdered 3 : 5-diiodothyronine accordingly was covered with diphenylamine (20 parts) and heated in a metal-bath, the air in the flask being displaced by a current of dry hydrogen; the first evolution of gas was perceptible at about 190°; from this point the temperature of the bath was slowly raised, until, after about 45 minutes a temperature of 240° had been attained; by this time the whole of the amino-acid had passed into solution and evolution of gas had ceased. The light brown solution in the flask was allowed to cool to about 70° and was then diluted with benzene and light petroleum; separation of the amine commenced at once and was complete after some hours' standing in the cold. The somewhat pigmented crystalline powder was filtered and well washed with light petroleum; it was then dissolved in dilute alcohol with the aid of a little sulphuric acid, the solution was boiled with charcoal, filtered, and treated with excess of ammonia. The amine so obtained was practically pure, though still faintly coloured; the yield was 70–80 % of the theoretical. For complete purification the amine was converted into the sparingly soluble sulphate by dissolving in much boiling dilute sulphuric acid and allowing the solution to cool. The sulphate was filtered off, redissolved in dilute alcohol, and the base precipitated from the hot solution by addition of ammonia. The pure compound formed small colourless rhomboidal crystals, melting at 243–245°. It was fairly easily soluble in alcohol, but insoluble in the other common organic solvents and in water.

*Analysis.* 0.98 mg. required 4.85 cc. *N*/200 thiosulphate [Kendall, 1914].  
20.2 mg. gave 0.546 mg. N (micro-Kjeldahl).

			I	N
Found	...	...	52.5	2.7
Calculated for	$C_{14}H_{13}O_2NI_2$		52.8	2.9

The *hydrochloride* formed glistening plates which were sparingly soluble in cold water, but more readily so in dilute alcohol; the substance melted with decomposition at 285–290°.

*Analysis.* 1.53 mg. required 7.05 cc.  $N/200$  thiosulphate [Kendall, 1914].  
29.3 mg. gave 0.790 mg. N (micro-Kjeldahl).

		I	N
Found	... ..	48.8	2.7
Calculated for $C_{14}H_{14}O_2NClI_2$		49.1	2.7

The *sulphate* formed long fine needles with similar solubilities.

*Thyroxamine.* This compound was first prepared by the direct decarboxylation of thyroxine according to the method described above; it was impossible, however, to avoid a good deal of decomposition of the thyroxine, with liberation of free iodine, and the yield of the amine was unsatisfactory. Better results were subsequently obtained by the iodination of 3 : 5-diiodothyronamine. The latter compound (1 part) was dissolved in pure methyl alcohol (500 parts) to which was added concentrated aqueous ammonia (10 parts); the cold solution was then treated gradually with a concentrated (2.5  $N$ ) solution of iodine in potassium iodide in the theoretical amount. When the addition of the iodine was complete the solution, which now had a yellow tinge, was concentrated, when the amine separated as a heavy crystalline powder. This was filtered off and purified by solution in 70 % alcohol with the addition of a little mineral acid, followed by precipitation of the base with ammonia from the hot solution. Repetition of this process once or twice gave a product which was analytically pure, and which formed small colourless needles melting at  $207^\circ$  with decomposition.

*Analysis.* 50.3 mg. gave 64.3 mg. AgI.  
22.8 mg. gave 0.425 mg. N (micro-Kjeldahl).

		I	N
Found	... ..	69.1	1.9
Calculated for $C_{14}H_{11}O_2NI_4$		69.3	2.0

The *sulphate* and *hydrochloride* were both very sparingly soluble in water; they were soluble in dilute alcohol but did not crystallise well from this solvent.

The *chloroacetate* was obtained by dissolving thyroxamine in a warm aqueous solution of chloroacetic acid and allowing to cool. It formed fine hair-like needles, which were fairly readily soluble in warm water (in which, however, a clear solution could not be obtained owing to dissociation), and could be recrystallised from water containing a little chloroacetic acid. The substance darkened and melted at  $152^\circ$ .

*Analysis.* 54.3 mg. gave 0.858 mg. N (micro-Kjeldahl).

		N
Found	... ..	1.6
Calculated for $C_{16}H_{14}O_4NCl_4$		1.7

#### REFERENCES.

- Abderhalden and Gebelstein (1926). *Z. physiol. Chem.* **152**, 125.  
 Anschütz and Bertram (1904). *Ber. deutsch. chem. Ges.* **37**, 3971.  
 Gaddum (1927). *J. Physiol.* **64**, 246.  
 Johnson and Daschavsky (1925). *J. Biol. Chem.* **62**, 725.  
 Kendall (1914). *J. Biol. Chem.* **19**, 251.

# CLXXX. THE PHOSPHATASES OF MAMMALIAN TISSUES.

## II. PYROPHOSPHATASE.

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*(Received October 31st, 1928.)*

NEUBERG and WAGNER showed in 1926 that both diphenyl orthophosphate and diphenyl pyrophosphate, in aqueous solution in the form of their potassium salts, were hydrolysed practically completely, both by the phosphatase of takadiastase and by that of horse kidney, to salts of orthophosphoric acid [1926]. Neuberger and Jacobsohn [1928] have also shown that the potassium salt of di-*o*-cresol pyrophosphate is similarly hydrolysed by both of these phosphatases, and that potassium di-*m*-cresol pyrophosphate and potassium di- $\alpha$ -naphthol pyrophosphate are hydrolysed by takadiastase (kidney extract not mentioned). Both kidney extract and takadiastase (prepared from *Aspergillus oryzae*) contain, therefore, a pyrophosphatase. Lohmann [1928] has recently stated that pyrophosphates are present in muscle, and are hydrolysed to orthophosphates when the hashed muscle is incubated in sodium bicarbonate solution. This production of orthophosphate has hitherto been very generally ascribed to the hydrolysis of "lactacidogen," believed by Embden and others to be a hexosephosphate. If Lohmann's claim is substantiated, it follows that the greater part of the so-called "lactacidogen" phosphorus is not originally present as hexosephosphate at all. Still another factor is thus introduced into the problem of the complex rôle played by phosphate in the biochemical changes associated with the contraction and metabolism of muscle. His findings also give a new importance to the presence of pyrophosphatase in the tissues. It becomes of interest to know whether there is any relation between the enzyme which hydrolyses pyrophosphate and that phosphoric esterase, widely distributed in the tissues, which hydrolyses glycerophosphate, hexosediphosphate and nucleotide [Kay, 1928], and many other orthophosphoric esters.

### EXPERIMENTAL.

Pure sodium pyrophosphate was prepared by heating pure disodium hydrogen phosphate to a red heat in a platinum dish. So prepared, its aqueous solution gave a white precipitate (without the slightest trace of yellow) with silver nitrate and contained, as determined by the Briggs or the Bell-Doisy

method, no orthophosphate. (A commercial sample of "pure" potassium pyrophosphate was found to contain 10 % of orthophosphate.) Although quite stable in neutral or alkaline solution, preliminary experiments indicated that the salt was very slowly hydrolysed at room temperature by the concentration of acid present in the determination of orthophosphate by the Briggs method. Under the conditions used by me the hydrolysis was at the rate of 0.016 mg. P in  $\frac{1}{2}$  hour, starting with 2.20 mg. pyrophosphate-P in the reaction flask, which contained the usual Briggs reagents, made up to 25 cc. Thus about 0.7 % of the original pyrophosphate was hydrolysed at room temperature by  $N/2$   $H_2SO_4$  in  $\frac{1}{2}$  hour; this rate was linear for the first 2 hours at least. On this basis a correction<sup>1</sup> was applied where necessary. It is probable that the Briggs method, with the necessary correction, would prove useful in determining the rate of decomposition of pyrophosphate by acids, etc., under various conditions of temperature and concentration.

Both sodium and potassium pyrophosphate were found to be readily hydrolysed to orthophosphate by various tissue extracts. Pyrophosphate must be almost unique as an example of a substance stable to a bright red heat, yet capable of enzymic decomposition.

#### *Optimum $p_H$ for pyrophosphatase.*

This has been determined for the pyrophosphatase present in extracts of kidney cortex, duodenal mucosa, bone and lung of the cat, the kidney of the pig, and the bones of a young rat. In all cases it lies between  $p_H$  7.2 and 7.8 (in Palitzsch's borax-boric acid buffers), usually about 7.6 (Table I).

Table I. *Optimum  $p_H$  for action of pyrophosphatase.*

Palitzsch's borax-boric acid buffers used, duration of hydrolysis 2 hours at 37.5°. Figures in mg. orthophosphate-P produced in 10 cc. of reaction mixture.

$p_H$	Source of enzyme			
	Cat's kidney extract, fresh, 1/20	Pig's kidney extract, 1 year old, 1/20	Cat's duo- denal extract, 1/160	Young rat's bone extract, fresh, 1/20
6.8	0.092	0.089	0.074	0.245
7.1	0.104	0.111	0.094	0.303
7.36	0.114	0.114	0.117	0.366
7.6	0.126	0.117	0.086	0.403
7.9	0.123	0.111	0.059	0.387
8.2	0.104	0.088	0.034	0.341
8.5	—	0.061	—	0.245

It will be noticed that the optimal activity of pyrophosphatase at about  $p_H$  7.6 is in a much more physiological range than that of the orthophosphoric esterase ( $p_H$  8.8–9.3).

<sup>1</sup> In view of this slow hydrolysis of pyrophosphate by the Briggs reagents, and Lohmann's finding of relatively large quantities of pyrophosphate in fresh muscle, it may be necessary to apply a correction, varying in magnitude with the exact conditions, to the quantitative figures obtained for "phosphagen" in muscles by various workers, who have estimated it by the difference between inorganic phosphate determined by magnesia precipitation (or some similar method) in alkaline solution and by the Briggs colorimetric method (or some similar method) in acid solution.

*Distribution in the tissues.*

Extracts of the following tissues (arranged in order of their activity) were found to be hydrolytically active on solutions of pure sodium pyrophosphate in borate buffers: duodenal mucosa<sup>1</sup> (most active), growing bone, kidney, lung, liver, adult bone (least active). Muscle extract, blood-plasma and extract of gastric mucosa also contained a weak pyrophosphatase. The extracts were made as previously described [Kay, 1928]. Boiled extracts showed no hydrolytic activity. The active extracts, tested against glycerophosphate solutions of the same concentration of P per cc. as in the pyrophosphate solution, gave the usual optimum for the former substrate of  $p_H$  8.8–9.3. The order of hydrolytic activity displayed by the various tissue extracts was the same for glycerophosphate at  $p_H$  8.9 as for pyrophosphate at  $p_H$  7.6. (Such extracts hydrolysed pyrophosphate at  $p_H$  8.9, and glycerophosphate at  $p_H$  7.6, but only very slowly.) Usually with the more active extracts the amount of orthophosphate-P produced at the optimal  $p_H$  of 7.6 from pyrophosphate solutions was about half of that produced from glycerophosphate solutions at  $p_H$  8.9, *i.e.* the ratio

$$\frac{\text{amount orthophosphate-P produced from excess glycerophosphate at } p_H \text{ 8.9}}{\text{amount orthophosphate-P produced from excess pyrophosphate at } p_H \text{ 7.6}}$$

was usually found to be approximately constant from one tissue to another.

The hydrolysis of pyrophosphate by tissue extracts is brought about, therefore, by an enzyme whose distribution in the tissues appears to be similar to that of glycerophosphatase (= hexosediphosphatase = guanine nucleotidase), and it seems likely that the same phosphoric esterase is responsible. The reverse reaction of enzymic synthesis of phosphoric esters by tissue extracts I have shown to take place in the case of glycerophosphate [1928] and glycerophosphate (hydroxyethyl phosphate) by actually isolating the synthesised esters as the pure barium salts, but several attempts to accomplish the synthesis of pyrophosphate along similar lines from solutions of orthophosphates have so far been without success; the strong salt solutions used flocculate and inactivate the enzyme.

## SUMMARY.

Pyrophosphatase is widely distributed in mammalian tissues; its distribution is similar to that of the orthophosphoric esterase previously described, but its zone of optimal activity is between  $p_H$  7.2 and 7.8, as against  $p_H$  8.8–9.3 for the latter enzyme.

## REFERENCES.

- Kay (1928). *Biochem. J.* **22**, 855.  
 Lohmann (1928). *Naturwiss.* **16**, 298.  
 Neuberg and Jacobsohn (1928). *Biochem. Z.* **199**, 498.  
 Neuberg and Wagner (1926). *Biochem. Z.* **171**, 485.

<sup>1</sup> The great activity of intestinal extracts would suggest that pyrophosphates taken by mouth are probably hydrolysed to orthophosphates before absorption from the gut.

# CLXXXI. METABOLISM IN SCURVY. II.

## THE NITROGEN ABSORPTION AND RETENTION OF GUINEA-PIGS.

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*Report to the Medical Research Council.*

*(Received November 1st, 1928.)*

THE object of this investigation was to ascertain whether the pathological changes in the guinea-pig which produce the syndrome of scurvy are connected with a disturbance in the absorption or in the retention of nitrogen. When a healthy guinea-pig of about 300 g. is placed on a scorbutic diet well balanced in other respects and is kept under hygienic and comfortable conditions it will grow and consume its normal requirements for about 21 days or even longer, although it has been suffering from scurvy for some days. After this stage the appetite diminishes and loss in weight is recorded owing to the aggravated condition of the animal caused by the progress of the disease. In the case of guinea-pigs kept in metabolism cages the diminished consumption of food may manifest itself a few days earlier owing to the greater discomfort caused by the scorbutic condition in the exertion of reaching the food. Animals maintained on scorbutic diets which are also deficient in other respects, such as a one-sided oats diet or a balanced mixture composed mainly of constituents which are not natural for the guinea-pig and consequently not very palatable, will begin to consume less of their food quite early. It is evident, therefore, that the character of the diet is of great importance in dietetic experiments in which the bearing of scurvy on metabolism is studied. The diet used in this investigation has proved to be suitable for this purpose. Despite the less comfortable conditions of the metabolism cage as compared with the ordinary cage, the animals began showing a tendency to consume less food at a time when the early symptoms of scurvy are usually in evidence and consequently any disturbance in the absorption and retention of nitrogen associated with the onset of scurvy, or the development of the disease previous to the manifestation of obvious symptoms, would have been reflected at a time when the consumption of food was normal. Our results suggest that such disturbance does not take place. Whether a deranged metabolism of the

absorbed nitrogen is caused by the vitamin deficiency is being studied, alongside similar problems with a view of throwing some light on the mode of action of the antiscorbutic factor.

### *Experimental procedure.*

Young growing guinea-pigs were employed since the quickly developing animal was considered to be more sensitive in manifesting a change in its nitrogen balance. The animals were kept in modified Hopkins metabolism cages, in a room the temperature of which never fell below 65° F. The cages which were 25 cm. in diameter were fitted with a detachable zinc floor perforated with 2 cm. holes, thus enabling the faeces to drop out of the cage fairly freely. At the end of the tunnel which formed an arm of the cage there was a frame in which a shutter, separating the cage from the food receptacle, was introduced. In the centre of the shutter was a round hole of such a size as to permit the animal free access to the food but preventing it from dragging any food into the tunnel or the cage. A glass separator, described by Gross and Connell [1923], was attached to the collecting funnel. Before the commencement of an experiment several specially chosen guinea-pigs of suitable weight were placed separately in the metabolism cages and were watched for some time. Only those animals which showed the capacity of adapting themselves without much trouble to this existence were selected for investigation.

The food mixture was made up as follows. 325 g. of bran were thoroughly mixed with 190 g. of barley meal and enough warm water was added to the mixture to make it damp and give it a convenient consistency. 280 g. of middlings and 180 g. of fish meal were then added and the whole mixture was rubbed in small quantities between the hands in order to ensure a uniform distribution. Each batch of food, which was prepared for several days, was analysed for its nitrogen and dry matter content and was kept in an air-tight tin receptacle in the cold room. Bi-weekly nitrogen and dry matter determinations have shown that the composition of each batch of food remained constant. Besides this food mixture, which was given *ad lib.*, the animal received a definite quantity of milk every day, delivered by hand, so that the quantity of liquid administered per day was always the same. The milk was made up from an analysed dried full-cream milk stock powder which was kept under the same conditions as the food mixture and which, by frequent analyses, was shown to keep a constant composition. 31.250 g. of this powder (containing 97.15 % of dry matter) was dissolved in 250 cc. of water and kept in a well-stoppered bottle in the cold room. The uniformity of the nitrogen content was assured by analysing each batch of milk. 40 cc. of milk was administered to the animal daily in 10 cc. doses. On Sundays two doses of 10 cc. each of twice the normal strength of milk were given. 30 g. of the food mixture was weighed out every morning into a tared glass vessel which was placed in the food compartment. The following morning at the same time the food was weighed back. After careful observation we have satisfied ourselves that no

visible particle of food ever penetrated the tunnel or cage during the course of the experiments. The dry matter and nitrogen content of the residual food mixture were determined as soon as possible after collection.

The urine was collected every morning at the same time in a weighed flask containing a known quantity of nitrogen-free acid—tartaric acid in the case of guinea-pig No. 1 and phosphoric acid in the remaining animals—and was weighed immediately after collection. The receptacles in which the faeces were received had cardboard covers which were kept damp in order to check evaporation as much as possible. After ascertaining the wet weight of the faeces the material was dried in an oven at 100° to brittle consistency, when it was ground and aliquot portions were taken for the determination of dry matter and of nitrogen.

The dry matter content of the food, oven-dried faeces, and evaporated urine was determined by drying the material at 78° in a vacuum over phosphorus pentoxide to constant weight. The nitrogen content was determined by a micro-method in which the material was oxidised with boiling concentrated sulphuric acid in the presence of anhydrous potassium sulphate and copper sulphate and the resulting ammonia after addition of sodium hydroxide was aspirated into standard acid. 0.5 g. was employed in the case of the food mixture and of the oven-dried faeces and 1.0 g. of the urine as collected. The carbon was determined on the vacuum-dried urine by the usual combustion method, 0.1 g. of material being used in the determination.

#### *Discussion of results.*

*Guinea-pig No. 1* (Tables I and IV). The experiment on this animal was divided into four periods. The first period was devoted to the investigation of the suitability of the technique. It commenced on Feb. 1, 1928 and lasted 28 days. During this period the animal received the basal diet *ad lib.*, 40 cc. of milk and 5 cc. decitrated lemon juice *per diem*. The antiscorbutic dose contained about 0.5 g. of solids, mostly sugar. The nitrogen administered in this way was less than 1 mg. per day and was consequently outside the limits of accuracy of the method. The quantity of the food mixture consumed by the animal was of the same magnitude as that eaten by a similar animal in an ordinary cage, which with the help of the above antiscorbutic dose was found by one of us to promote normal growth and excellent health in guinea-pigs of this age. The adequacy of this basal diet for the guinea-pig is, therefore, apparent. During the 28 days of this period the animal gained about 50 g.—a gain which is, perhaps, rather less than the normal but which may be considered quite satisfactory in view of the lesser comfort of the metabolism cage. It will be seen that the nitrogen balance during this period was definitely positive and more or less commensurate with the growth. The nitrogen intake (average 614 mg.) varied roughly from 500 to 700 mg. per day and was controlled by the amount of the basal mixture consumed daily and by the variation in the nitrogen content of the different batches of this mixture. The average daily



Table I.

Date	Weight (g.)	Dry food (g.)	Food-N (mg.)	Milk-N (mg.)	Total N intake (mg.)	Weight of urine (g.)	Urine-N (mg.)	Weight of faeces (g.)	Dry weight of faeces (g.)	% of dry matter in faeces	Faecal N (mg.)	Faecal N % of total N intake	Total N output (mg.)	Nitrogen balance (mg.)
ii. 1	305	15.52	600.7	84	684.7	26.233	449	6.202	4.462	71.9	98	14.3	557	+ 138
2	310	14.65	564.7	84	648.7	32.300	510	6.579	4.159	63.2	104	16.0	614	+ 35
3	310	15.64	615.0	84	699.0	25.152	443	10.021	4.128	41.2	100	14.3	543	+ 156
4	315	14.72	421.6	84	505.6	23.345	293	12.188	6.186	50.7	149	29.4	442	+ 64
5	315	16.66	453.6	84	537.6	23.305	378	3.304	1.942	58.8	50	9.3	428	+ 110
6	310	15.54	431.0	84	515.0	20.471	376	10.138	5.216	50.5	150	29.1	526	- 11
7	320	18.09	510.8	84	594.8	32.330	394	12.960	4.810	37.2	125	21.0	519	+ 76
8	322	16.01	444.8	84	528.8	26.286	435	5.096	4.455	89.4	102	19.3	537	- 8
9	325	18.07	637.0	84	721.0	26.286	423	9.078	4.373	45.9	97	13.5	520	+ 201
10	325	16.42	567.2	84	631.2	28.194	458	12.485	5.580	44.6	137	21.0	595	+ 56
11	327	13.84	487.3	84	571.3	18.640	476	8.105	4.179	51.5	95	16.6	571	0
12	322	9.88	373.8	84	467.8	22.618	606	8.545	4.568	53.4	86	18.8	692	- 235
13	320	14.85	522.6	84	606.6	27.060	592	7.128	4.032	56.5	92	15.2	684	- 77
14	332	16.84	601.0	84	685.0	25.493	381	9.210	4.434	48.1	103	15.0	484	+ 201
15	340	17.87	642.2	84	726.2	20.170	428	12.020	4.187	34.8	99	13.6	527	+ 199
16	345	17.68	620.2	84	704.2	18.482	319	12.157	4.103	33.7	92	13.1	411	+ 293
17	347	16.82	590.7	84	674.7	22.820	370	11.920	5.099	42.7	114	16.9	484	+ 191
18	350	13.60	476.2	84	560.2	25.469	436	10.099	4.553	45.1	108	19.3	544	+ 16
19	345	13.60	473.2	84	557.2	16.630	542	12.700	6.359	50.1	140	25.1	682	- 125
20	335	16.71	538.2	84	622.2	19.022	579	8.819	4.335	49.1	107	17.2	686	- 64
21	342	19.01	607.4	84	691.4	21.541	497	7.902	4.006	50.7	89	12.9	586	+ 105
22	360	20.21	694.8	84	778.8	29.985	488	6.492	3.606	55.5	86	11.0	574	+ 205
23	360	19.73	660.5	84	744.5	35.361	597	13.082	5.553	42.3	128	17.2	725	+ 19
24	360	19.89	674.5	84	758.5	27.229	539	11.424	4.937	43.2	117	14.9	656	+ 102
25	355	18.14	570.8	84	654.8	25.271	403	6.053	3.747	61.9	90	13.7	493	+ 162
26	360	10.84	339.5	84	423.3	14.345	379	10.652	5.521	51.8	127	29.9	506	- 83
27	340	14.28	458.3	84	542.3	20.674	498	11.597	6.352	54.7	158	29.1	656	- 114
28	355	8.92	308.7	84	392.7	21.126	250	8.331	3.682	44.2	84	21.4	334	+ 59
29	360	9.45	329.6	42	371.6	26.496	288	6.080	2.297	37.7	51	13.7	339	+ 33

iii. 1	345	9.84	337.9	42	379.9	14.712	310	5.445	2.297	42.1	53	13.9	363	+ 16.
2	342	10.00	340.0	42	382.0	20.538	318	5.666	2.480	43.7	58	15.1	376	+ 6
3	350	10.34	356.0	21	377.0	16.315	447	6.884	3.222	46.8	77	20.4	524	- 147
4	335	10.31	354.0	21	375.0	27.503	491	7.412	3.052	41.2	65	14.7	546	- 171
5	325	10.32	355.3	42	397.3	24.512	432	7.511	2.895	39.5	64	16.1	496	- 99
6	325	7.50	258.4	21	379.4	18.375	333	7.104	2.831	38.3	79	28.3	412	- 133
7	320	7.51	257.6	21	278.6	17.175	314	7.373	3.389	45.9	79	28.3	393	- 114
8	315	19.94	712.8	84	796.8	27.388	537	12.442	5.232	42.1	119	14.9	656	+ 141
9	315	18.73	669.2	84	763.2	27.715	506	9.118	5.479	60.1	132	17.5	638	+ 115
10	320	15.24	537.2	84	621.2	25.119	491	8.230	4.109	49.9	97	15.6	588	+ 33
11	320	13.15	461.5	84	545.5	15.052	366	9.516	5.835	61.3	142	26.0	508	+ 37
12	315	17.92	625.6	84	709.6	27.123	648	7.647	5.322	69.5	124	17.4	772	- 72
13	320	16.01	564.2	84	648.2	29.226	406	6.492	3.309	50.9	77	11.9	483	+ 165
14	325	12.89	458.9	84	542.9	27.806	260	7.590	4.440	58.5	104	19.2	364	+ 179
15	325	18.20	655.7	84	739.7	24.102	459	6.119	4.431	72.4	102	13.8	561	+ 179
16	332	16.72	603.8	84	687.8	23.583	511	8.851	4.939	55.8	115	16.7	626	+ 62
17	330	17.51	615.8	84	699.8	24.635	597	5.146	4.701	91.2	107	15.3	704	- 4
18	325	14.23	498.7	84	582.7	23.490	496	6.514	3.950	60.7	74	12.7	570	+ 13
19	325	11.55	475.3	84	559.3	24.988	517	3.917	2.680	68.3	64	11.5	581	- 22
20	327	17.93	608.5	84	692.5	24.082	593	7.548	4.454	58.9	99	14.3	682	0
21	332	16.83	602.8	84	686.8	25.160	474	8.964	5.266	58.7	106	15.4	580	+ 107
22	345	17.32	622.6	84	706.6	23.504	459	8.457	5.428	64.1	105	14.9	564	+ 145
23	350	15.74	561.0	84	645.0	28.182	541	7.891	4.446	56.3	109	16.9	650	- 5
24	352	13.60	649.8	84	633.8	19.413	464	7.987	5.216	65.2	118	18.6	582	+ 52
25	340	9.81	355.7	84	439.7	16.744	414	5.976	3.262	54.5	74	16.8	488	- 48
26	330	10.34	367.8	84	451.8	17.902	448	7.351	4.007	54.5	91	20.1	539	- 87
27	332	5.66	182.6	84	266.6	38.402	363	8.710	4.187	48.1	77	28.8	440	- 173
28	315	NH	NH	84	84.0	25.304	349	1.727	1.628	94.1	37	44.1	386	- 302
29	285	NH	NH	84	84.0	17.514	264	NH	NH	—	NH	—	264	- 180
30	270	NH	NH	NH	NH	NH	NH	NH	NH	—	NH	—	NH	—

Table II.

Date	Weight (g.)	Dry food (g.)	Food-N (mg.)	Milk-N (mg.)	Total N intake (mg.)	Weight of urine (g.)	Urine-N (mg.)	Weight of faeces (g.)	Dry weight of faeces (g.)	% of dry matter in faeces	Faecal N (mg.)	Faecal N % of total N intake	Total N output (mg.)	Nitrogen balance (mg.)	C/N ratio
iv. 12	435	16.71	568	84	652	33.03	403	7.13	4.57	64.1	111	17.0	614	+138	0.96
13	445	15.62	524	84	608	27.76	486	7.39	4.03	54.5	93	15.3	579	+ 29	0.93
14	445	16.17	545	84	629	20.50	521	9.48	4.10	43.3	100	15.8	621	+ 8	0.97
15	430	12.63	423	84	507	17.87	412	12.37	5.33	43.1	115	22.7	527	- 20	0.80
16	430	13.30	444	84	528	18.31	512	8.62	3.98	46.2	100	18.9	612	+ 84	0.99
17	430	14.50	486	84	570	22.84	424	13.85	5.38	38.8	128	22.4	552	+ 18	0.95
18	435	15.74	530	84	614	29.22	481	14.64	5.33	36.4	113	18.5	594	+ 20	0.98
19	437	16.17	644	84	628	23.18	382	14.26	5.75	40.3	130	20.7	512	+116	0.99
20	430	15.39	517	84	601	21.61	323	10.04	5.12	50.0	120	19.9	443	+188	0.84
21	420	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	410	8.78	293	84	377	17.26	381	10.41	4.13	39.8	95	25.2	476	- 99	1.10
23	410	14.07	469	84	553	18.06	296	10.18	3.87	38.0	101	18.2	397	+156	1.20
24	415	13.90	472	84	556	17.37	294	12.73	4.73	37.2	108	19.4	392	+164	1.53
25	417	13.87	466	84	550	19.71	321	12.62	5.78	45.8	145	26.3	466	+ 84	1.18
26	420	13.07	444	84	528	25.48	390	9.21	4.68	50.9	122	23.1	512	+ 16	1.09
27	420	10.92	397	84	451	27.94	315	11.31	5.22	46.2	126	27.9	441	+ 10	1.23
28	415	6.65	233	84	317	16.28	281	12.12	5.71	47.1	134	42.2	415	- 98	1.12
29	405	6.25	207	84	291	13.53	275	3.27	2.52	76.8	79	27.1	354	- 63	1.02
30	390	6.96	199	84	283	16.74	282	10.13	5.97	59.1	145	51.2	427	-144	1.34
v. 1	395	4.69	158	84	242	19.13	275	7.74	5.06	65.4	119	49.2	394	-152	1.22
2	397	3.12	104	84	188	26.94	358	5.94	3.90	65.7	90	48.4	448	-260	0.99
3	392	2.81	93	84	177	27.37	396	2.95	1.94	65.6	46	25.9	448	-260	0.92
4	375	NH	NH	84	84	22.77	262	1.82	1.02	56.0	25	29.9	287	-203	1.33
5	352	NH	NH	84	84	28.95	381	NH	NH	—	NH	—	381	-297	0.82

Table III.

Date	Weight (g.)	Dry food (g.)	Food N (mg.)	Milk-N (mg.)	Total N intake (mg.)	Weight of urine (g.)	Urine-N (mg.)	Weight of faeces (g.)	Dry weight of faeces (g.)	% of dry matter in faeces	Faecal N (mg.)	N % of total N intake	Faecal Total N output (mg.)	Nitrogen balance (mg.)	C/N ratio
vi. 15	355	12.39	451	84	535	19.14	286	7.14	4.87	68.2	124	23.2	410	+125	0.92
vi. 16	360	12.36	450	84	534	8.51	269	7.26	3.37	49.2	92	17.3	361	+173	0.80
17	360	14.06	510	84	594	22.44	293	8.39	4.49	53.5	118	19.8	411	+187	0.88
18	362	12.30	460	84	544	17.14	276	8.12	5.39	66.3	141	25.9	417	+123	0.85
19	365	14.04	514	84	598	21.58	324	9.10	4.32	47.5	114	19.1	438	+160	0.95
20	370	13.44	488	84	572	23.42	352	9.90	4.49	45.4	113	19.6	465	+107	0.97
21	370	7.39	271	84	355	22.44	262	8.95	4.36	48.7	108	30.4	370	-15	1.16
22	365	9.11	331	84	415	19.35	347	7.83	3.45	44.1	87	20.9	434	-19	0.83
23	360	8.43	303	84	387	20.62	380	6.39	2.80	43.9	67	17.3	447	-60	0.87
24	355	7.83	277	84	361	18.44	339	6.44	3.14	48.8	76	21.1	415	-54	0.84
25	345	7.14	258	84	342	19.22	326	8.41	3.62	43.0	93	27.2	419	-77	0.91
26	345	8.06	286	84	370	24.26	413	7.63	3.58	46.9	92	24.8	505	-135	0.79
27	345	12.56	462	84	536	22.10	370	7.50	3.53	47.1	92	17.2	462	+74	0.84
28	360	14.34	513	84	597	20.38	347	8.71	5.18	59.4	132	22.1	479	+118	0.84
29	365	14.47	523	84	607	24.81	428	8.94	4.49	50.3	117	19.3	545	+62	0.86
30	370	14.99	543	84	627	23.62	397	8.99	4.44	49.4	114	18.2	511	+116	0.78
vii. 1	365	9.63	342	84	426	20.10	340	9.38	4.06	43.3	105	24.7	445	-19	0.75
2	360	6.33	224	84	368	18.60	293	10.29	4.22	41.0	109	35.4	402	-94	0.83
3	350	7.68	277	84	361	22.01	328	9.90	3.64	37.1	96	26.6	424	-63	0.87
4	345	7.39	262	84	346	27.44	327	5.03	2.69	51.4	67	19.4	394	-48	1.09
5	340	3.60	130	84	214	25.06	351	4.18	2.17	51.8	56	26.2	407	-193	0.92
6	330	0.88	31	84	115	20.54	329	3.15	2.30	73.1	58	50.4	387	-273	0.83
7	325	0.61	22	84	106	19.36	292	3.03	1.72	56.6	44	41.5	336	-230	0.92
8	320	Nil	Nil	84	84	20.48	287	2.79	1.90	68.1	47	55.9	324	-250	0.98
9	305	Nil	Nil	84	84	21.54	305	2.50	1.01	40.4	25	29.7	330	-246	0.94
10	300	Nil	Nil	84	84	24.72	340	2.63	1.76	66.9	44	52.4	384	-300	0.90
11	295	Nil	Nil	84	84	21.98	314	2.63	1.46	72.6	36	42.8	350	-296	0.85
12	285	Nil	Nil	84	84	14.52	258	2.16	1.62	75.0	39	46.4	297	-213	0.83
13	285	7.49	270	84	354	16.44	277	4.03	2.28	56.0	52	14.7	329	+25	0.94
14	290	10.02	362	84	446	20.79	320	6.83	2.71	46.5	63	14.1	383	+63	1.08
15	305	12.53	452	84	536	22.74	307	8.91	4.44	49.8	113	21.1	420	+116	0.99
16	310	12.33	438	84	522	21.73	300	8.83	4.08	53.0	116	22.2	416	+106	1.01
17	310	11.78	424	84	508	20.20	272	9.01	4.42	49.1	105	20.7	377	+131	1.06
18	315	11.57	422	84	506	20.14	300	12.13	4.74	39.0	112	22.1	412	+94	0.94
19	317	12.36	447	84	531	20.42	289	12.70	5.58	43.9	136	25.6	425	+106	0.94
20	320	11.82	434	84	540	23.32	275	10.52	5.52	52.5	129	25.4	404	+157	1.17
21	322	12.86	456	84	540	16.26	236	13.41	6.21	46.3	147	27.2	383	+167	1.00
22	325	12.16	443	84	527	17.61	287	9.32	4.38	46.9	104	19.7	391	+136	0.93
23	327	11.67	423	84	507	20.40	331	9.85	3.86	40.2	87	17.2	418	+89	0.91
24	327	11.99	431	84	515	24.72	371	10.39	5.09	49.0	110	21.3	451	+34	0.89

dry matter evacuated in the faeces (4.6 g.) was roughly a fourth of that ingested in the food (15.7 g. of the food mixture and about 5 g. of the dry milk), whilst about a sixth of the nitrogen ingested was excreted in this way. In the second period the food, mainly the basal mixture, was restricted so that the animal lost in weight, the quantity administered daily being controlled by the weight of the animal. The same dose of decitrated lemon juice was given in this period as in the preceding one, thus keeping the animal fully protected from scurvy.

Table IV.

Period	...	...	I	II	III	IV
Dry food (g.)	...	...	15.7	9.3	16.6	14.1
Nitrogen intake (mg.)	...	...	614	357	678	449
Urine (g.)	...	...	24.8	20.7	25.2	22.6
Urinary nitrogen (mg.)	...	...	449	352	465	414
Dry faeces (g.)	...	...	4.6	2.9	4.8	4.3
Faecal nitrogen (mg.)	...	...	105	66	112	73
Faecal % N of total N intake	...	...	18.0	19.1	16.8	15.7
Nitrogen balance (mg.)	...	...	+60	-61	+101	-38

Period I. 28 days; basal diet + 5 cc. decitrated lemon juice.

" II. 8 days; restricted quantities of basal diet + 5 cc. decitrated lemon juice.

" III. 10 days; basal diet + 5 cc. decitrated lemon juice.

" IV. 12 days; basal diet + 5 cc. water.

In examining the figures of this period, it is seen, as one would expect, that there was a diminution in the amount of dry matter and nitrogen ingestion. The excretion of dry matter in the faeces and of nitrogen in the faeces and in the urine was accordingly diminished. The ratios, however, of the excreted dry matter or of nitrogen to those consumed did not alter to any great extent. The nitrogen balance of this period was, of course, negative. The eight days of restricted feeding were then followed by a recuperative period of 10 days, during which time the guinea-pig was fed as in period I. The figures obtained during this time resemble, in general, those of the first period. In the final period the decitrated lemon juice was discontinued and replaced by an equal volume of water. The animal was consequently receiving a scorbutic diet. During the first 9 days the guinea-pig progressed well, but at this stage it developed pneumonia, from which it died 4 days later. During the last 2 days it ceased consuming the food mixture. At the *post mortem* examination definite ridging of the costochondral junctions was found, but no other macroscopic scorbutic lesions were detected. It will be seen that during the 8 days of the scorbutic period, the intake and output of the food and nitrogen were proceeding more or less as in periods I and III, and the relationship of the output of the dry matter and of the nitrogen in the faeces to their intake is not very different from that in the other periods. The nitrogen balance was again roughly parallel to the growth of the animal. Only during the last 3 or 4 days was there a marked negative balance which was due to the very rapid premortal decline.

*Guinea-pig No. 2* (Tables II and V). After a preliminary period of 6 days on the basal diet and 5 cc. of decitrated lemon juice, the antiscorbutic was

replaced by an equal volume of water. During the first 10 days of the scorbutic period the animal consumed its food well but after that time the intake gradually diminished and after a further 6 days there was a cessation in the consumption of the food mixture, the animal dying 2 days later. At the *post mortem* examination pneumonia, slightly swollen knee joints, haemorrhages in the gastrocnemius and femoral muscles of both legs and slightly enlarged costochondral junctions were established. In examining the intake and output figures during the first 10 days of the scorbutic period, when the consumption of food was normal, no striking differences are found between them and those of the preliminary period. The output of dry matter and of nitrogen in relation to their intake is slightly higher but this is of an order to which no significance can be attached. As in the preceding animal, there was a very marked negative nitrogen balance during the decline in the premortal phase.

Table V.

Period	...	...	I	II
Dry food (g.)	...	...	14.8	13.5
Nitrogen intake (mg.)	...	...	583	519
Urine (g.)	...	...	23.4	22.3
Urinary nitrogen (mg.)	...	...	459	345
Dry faeces (g.)	...	...	4.5	4.9
Faecal nitrogen (mg.)	...	...	108	118
Faecal N % of total N intake	...	...	18.7	22.1
Nitrogen balance (mg.)	...	...	+16	+56

Period I. 6 days; basal diet + 5 cc. decitrated lemon juice.

„ II. 10 days; basal diet + 5 cc. water.

Table VI.

Period	...	...	I	II	III	IV
Dry food (g.)	...	...	13.0	9.9	0.6	11.5
Nitrogen intake (mg.)	...	...	562	440	104	499
Urine (g.)	...	...	17.9	21.8	21.0	20.4
Urinary nitrogen (mg.)	...	...	291	349	309	298
Dry faeces (g.)	...	...	4.5	3.8	1.7	4.5
Faecal nitrogen (mg.)	...	...	118	98	44	106
Faecal N % of total N intake	...	...	21.0	22.9	43.2	20.9
Nitrogen balance (mg.)	...	...	+153	-7	-249	+95

Period I. 5 days; basal diet + 5 cc. decitrated lemon juice.

„ II. 15 days; basal diet + 5 cc. water.

„ III. 8 days; 6 days on basal diet + 5 cc. water and 2 days on basal diet + 5 cc. decitrated lemon juice.

„ IV. 12 days; basal diet + 5 cc. decitrated lemon juice.

*Guinea-pig No. 3* (Tables III and VI). This animal had a preliminary period of 5 days, followed by a scorbutic period of 21 days, in which the decitrated lemon juice was replaced by water, and eventually by a curative period in which the decitrated lemon juice was resumed. In the very early part of the scorbutic period there was, for some unknown reason, a diminished consumption in the food mixture for a few days, but the animal soon resumed its normal consumption until the onset of the early symptoms of scurvy when the intake began diminishing as before until no consumption of the mixture was recorded. The diminution in the food consumption during the very early

stages of the scorbutic period has fortuitously proved of use for comparison with a short period when the consumption was of the same order between 10th and 15th day and when the animal was undoubtedly scorbutic. By examining the nitrogen balance of these two periods one finds a great similarity, namely, a small negative balance caused by an insufficient intake of nitrogen. There is no indication that the presence of incipient scurvy made any difference in this respect. Again, when one examines the average figures for the first 15 days of the scorbutic period (Table VI, period II) one finds that in spite of the somewhat diminished intake of the food and of nitrogen, the ratios of the output of total solids and of nitrogen in the faeces to their intake is not appreciably different from those of the first and last periods. Between the 15th and 21st day of the scorbutic period and during the first 2 days of the last period, owing to the extremely small consumption of the basal mixture, the above ratios are abnormally high and the balance is markedly negative. The figures of the last period following the recovery from scurvy are very similar to those of the preliminary period.

#### CONCLUSIONS.

It will be seen from the above results that, in spite of all care, untoward complications, such as the intervention of pneumonia, could not be prevented. Disconcerting as this may be, it does not influence the results in such a way as to prevent them from affording the information which they were intended to yield, namely, whether there is any association between the onset of scurvy and a change in the absorption and retention of nitrogen. It was assumed that if this were the case it would manifest itself at an early stage of the animal's existence on a scorbutic diet, since it has been shown that macroscopic scorbutic lesions are in evidence approximately 10 days after the guinea-pig has been placed on the scorbutic diet, whilst microscopic changes may be observed even earlier [Zilva and Wells, 1919; Höjer, 1924]; in fact, one of us has had the opportunity of observing small subcutaneous haemorrhages in some cases as early as the 6th day. Daily observations on the intake and output of nitrogen would, therefore, bring to light even an abrupt change of short duration before this could be vitiated by the superimposition of the changes due to starvation. In analysing the above results one does not find even a suggestion that this is the case. In turning our attention to the figures dealing with the retention of nitrogen we see that in all the experiments the nitrogen balance is generally controlled by the amount of food consumed and in consequence by the growth of the animal in some instances, and is independent of the scorbutic condition of the animal. In the premortal phase or when the scurvy is acute, the balance is markedly negative, not because of the scurvy but as a consequence of starvation caused by the disease, either alone or complicated with pneumonia. Were the syndrome of scurvy a sequel to a disturbed nitrogen retention caused by the vitamin deficiency it would become evident not much later than the 10th day. The accidental similarity of the food intake of guinea-

pig No. 3 between 20th and 27th June and between 30th June and 5th July shows further that this is not the case. Similarly, the results fail to show a diminished assimilation of nitrogen. This is particularly evident from the tables of averages which show that there is no striking difference in the relation between the faecal nitrogen and the total nitrogen intake of the scorbutic and normal periods. A difference is observed only when the intake of the food mixture is almost nil, as occurs during the few days preceding death or during period III of guinea-pig No. 3, which is evidently the effect and not the cause of scurvy. The C/N ratios in the urine of guinea-pigs 2 and 3 were not concordant; in the first animal there was a definite increase in the ratio soon after the animal was placed on the scorbutic diet. This increase persisted until the death of the animal. No such deviation was observed in the case of guinea-pig No. 3.

As far as we are aware the only investigations which have been carried out on the nitrogen balance of scorbutic guinea-pigs in relation to normal animals have been recorded by Baumann and Howard [1917] and by Jarussowa [1928]. The former investigators have noted a high negative balance during the scorbutic period but, as their animals consumed an insufficiency of nitrogen and of calories during this period, a negative balance was to be expected. Jarussowa tried to overcome these difficulties. Briefly, her procedure consisted in forcibly feeding the guinea-pigs, which were kept in a metabolism cage, with powdered oats in the form of pills and boiled cabbage juice. Figures for a series of periods, but not daily figures, are given by her. It transpires from these data that the animal maintained its initial weight of 396 g. more or less steadily for 24 days, after which time it began losing weight rapidly. During these 1-24 days all the periods show a small positive balance. The following two periods are marked by an increasing and significant loss (18.2 %) in weight with a corresponding negative balance which lasted to the end of the experiment. The total daily nitrogen intake except for the last 2 days lay in the neighbourhood of 500 mg., the daily output in the urine was a little less than 400 mg. and showed little variation, whilst the nitrogen output in the faeces was of the order 100 mg. per day for the first 18 days with about a 20 % increase during the remainder of the experiment. This picture is not unlike the one we have obtained. We do not, however, agree with Jarussowa that "...während der Skorbutentwicklung geht die Stickstoffbilanz von einer positiven Grösse zu einer negativen über..." Scurvy in the young guinea-pig does not develop on the 24th day, when the change in the nitrogen balance occurred in these experiments. At this time the disease is of such severity as to disturb seriously all the functions of the animal organism. For the same reason we do not think that the higher nitrogen content of the faeces during the last 12 days of the experiment, even if it be a regular feature, which our experiments make us doubt, is caused directly by scurvy. Jarussowa also observed that the C/N ratio of the urine doubled between the 18th and 29th day and then became normal again until the end of the experiment. This



observation taken in conjunction with the discordant results we have obtained in this respect calls for further experiments in this direction. It seems, however, unlikely, from both investigations that the development of scurvy is associated with an increased C/N ratio in the urine.

#### SUMMARY.

There are no indications of a disturbed absorption or retention of nitrogen during the early stages of the development of scurvy in growing guinea-pigs. The nitrogen balance becomes negative only when the intake of food is diminished as a consequence of the disease. Although the results obtained were not concordant, it is unlikely that the C/N ratio of the urine of young guinea-pigs is disturbed by the onset of scurvy.

#### REFERENCES.

- Baumann and Howard (1917). *Amer. J. Med. Science*, **53**, 659.  
Gross and Connell (1923). *J. Physiol.* **57**, Proc. **ix**.  
Höjer (1924). *Acta Paediatrica*, **8**.  
Jarussowa (1928). *Biochem. Z.* **198**, 128.  
Zilva and Wells (1919). *Proc. Roy. Soc. Lond. B*, **90**, 505.

## CLXXXII. "HYPERVITAMINOSIS" AND "VITAMIN BALANCE<sup>1</sup>."

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*Report to the Medical Research Council.*

*From the Nutritional Laboratory, Cambridge.*

*(Received November 1st, 1928.)*

THE experimental observation that vitamins exert their normal physiological action when consumed in very minute doses leads naturally to the enquiry, "Have the vitamins any injurious effect when consumed in abnormally large amounts?" A similar, and probably closely related, question also arises, "Does an increased or diminished consumption of one vitamin affect the body's requirements for the others?" A great number of past writers have assumed, either tacitly or directly, that both these questions could be answered in the negative. In the present communication an attempt is made to collate and extend certain evidence to the contrary.

In regard to the water-soluble vitamins no evidence is as yet available to suggest that any ill-effects result from overdosing, though work with highly concentrated preparations has mainly been limited to the routine testing of minimal effective doses. In the case of the fat-soluble vitamins, on the other hand, several instances of supposed hypervitaminosis have been recorded, and several attempts have also been made to show that the fat-soluble and water-soluble requirements of the animal are to some extent interrelated.

In this country the earliest recorded work bearing on these questions is that of Hopkins [1923], who in the course of the Cameron lecture for 1923 mentioned that he had found that excess of cod-liver oil was often injurious to a rat unless "balanced" by an increased marmite (vitamin B) allowance. Somewhat later, in 1925, the work of Takahashi and his co-workers became generally known. To Takahashi is due the first suggestion that vitamins could have a lethal effect. Between 1922 and 1925 several papers had been published, in Japanese journals [1922, 1923], in which it was claimed that vitamin A (biosterin) had been isolated in a state of purity, and in the English article describing the substance [1925] it was reported that while daily doses of 0.004 mg. sufficed for the normal growth of rats, doses of 1.6 mg. invariably

<sup>1</sup> Certain conclusions here given were briefly summarised in the *Lancet* (1928), ii. 892.

<sup>2</sup> A particularised form of this question was put by Funk [1922]. "If, for example, the quantity of vitamin A is too little the requirements for vitamin B are perhaps increased. However, there are as yet no available data on this point." Funk, it will be noticed, is here suggesting the exact reverse of a balance, as understood in this paper.

caused failure in growth and death. Drummond and his collaborators [1925] however had been engaged in a patient research in the same field, and were able to contradict Takahashi's claim to have isolated the vitamin. Using carefully prepared concentrates no toxic action could be noticed in large doses, and it was suggested that the Japanese worker had been deceived by the presence in his preparation of poisonous by-products. Other investigators (e.g. Magliano [1925]) likewise stated that large excess of vitamin A or B, or of both, was harmless to a growing animal. In the face of such opposition interest in the problem naturally became small, and the possibility of vitamin excess, or vitamin balance, was generally held to be excluded.

From time to time, however, various isolated and little known observations have been recorded, mostly in somewhat inaccessible literature, which possibly have not received the attention they deserve. Mouriquand and Michel [1922], also Bezssonoff [1923], had spoken of the noxious effect of large doses of cod-liver oil in scurvy, and on somewhat similar lines Euler and Widell [1925] made the surprising claim that excess of cod-liver oil caused disturbances in the growth and bone formation of rats unless balanced by vitamin C<sup>1</sup>, a factor which is generally supposed to be unnecessary for the well-being of that animal. More recently Agduhr [1926] has reported degenerative changes in the heart after the feeding of excessive amounts of the oil to various animals, but these results have been attributed by Höjer [1926] mainly to an inadequate provision of the vitamin B complex. Hartwell [1927] and Sure [1927] have also carried out isolated work on the excessive feeding of cod-liver oil, special attention having been drawn to the injurious action of large excess of it during gestation.

In the foregoing work, it will be noted, cod-liver oil or its concentrates were used as sources of vitamin excess, and there was little to indicate whether vitamin A, vitamin D or some other constituent of the oil was responsible for the ill-effects observed. Recently irradiated ergosterol has largely replaced cod-liver oil as a source of vitamin D, and as a result of the investigations of Pfannenstiel [1927] and Kreitmair and Moll [1928] substantial evidence has accumulated to justify at least the suggestion that toxic properties may be attributed to this vitamin *per se*, though there is no reason to suppose that it is necessarily the only toxic factor present in vitamin concentrates. The second-named authors, after a thorough and painstaking research, have reported fatal effects resulting from massive doses in a variety of different species, with notable exceptions in the case of the hen and axolotl. They observed, *post mortem*, extensive depositions of calcium and a generalised sclerosis,—phenomena of much interest in view of the known action of vitamin D in increasing Ca (or P) absorption. That these records should be received not without some scepticism, or at least confusion, is understandable, for

<sup>1</sup> In further development of this line of thought Grant [1926] concludes that for normal development of the teeth it is necessary to have an adequate balance between the dietary calcium, vitamin C and vitamin D, excess of one magnifying the effect of deficiency of the others.

numerous other workers have recorded the apparent harmlessness to a human being of "large" doses of irradiated ergosterol (Kroetz [1927]; see also Havard and Hoyle [1928]) or to a rat up to 10,000 times the minimal effective dose (Rosenheim and Webster [1927]).

During the past year an attempt has been made in our laboratory to collate experimentally some of these scattered observations. A thorough survey is in progress, but we think it advisable to record the results so far obtained, which, in a general way, confirm definitely the idea of a harmful effect resulting from excessive intake of certain materials rich in fat-soluble vitamins.

#### EXPERIMENTAL.

All the experimental work recorded hereunder has been done on rats, either albino or piebald. Our procedure differed from that of Kreitmair and Moll in that growing animals were used, and that the vitamin preparations were administered mixed with the ration, instead of separately with forcible feeding when necessary or by intravenous injection.

##### I. *Excessive irradiated ergosterol.*

*Technique.* Young piebald rats, generally 40–50 g. in weight, were given "complete synthetic diets" containing irradiated ergosterol, in the concentrations 0.00001, 0.001 and 0.1 %. Animals from a single litter were distributed as evenly as possible. For controls, litter mates were given the same synthetic diet, but containing non-irradiated ergosterol and heated ergosterol in the same concentrations, and no ergosterol. Each rat in this experiment was isolated in a separate all-metal cage having a netted floor of somewhat coarse mesh.

*Diets.* The diet consisted of:

200 g. purified caseinogen,  
600 g. rice starch,  
50 g. salt mixture,  
190 cc. arachis oil;

the last ingredient containing the appropriate quantity of irradiated, non-irradiated, or heated ergosterol as the case might be. The irradiated ergosterol was supplied to us by Messrs British Drug Houses<sup>1</sup>. The heated ergosterol was prepared by leaving non-irradiated ergosterol in an open dish for 3 hours at a temperature slightly above the melting point. Each animal received in

<sup>1</sup> Messrs British Drug Houses have provided us with the following details of their procedure. A 0.5 % solution of ergosterol in absolute alcohol was subjected to ultra-violet radiation in a thin layer in the absence of oxygen, except that no attempt was made to remove dissolved oxygen from the alcohol. The duration of the exposure was 15 minutes. The solution was concentrated to a small bulk at a temperature not exceeding 40° with the use of a vacuum with a small stream of nitrogen. The concentrated solution was cooled and allowed to stand, for unchanged ergosterol to crystallise out. 14.9 % of the ergosterol originally taken was removed unchanged. The solution was further concentrated as before, transferred to a desiccator and the remainder of the solvent removed *in vacuo* at room temperature. We are informed by Messrs British Drug Houses that a different procedure is employed by them for their commercial preparation of

addition two drops of cod-liver oil daily, and 0.75 cc. of an alcoholic extract of marmite<sup>1</sup> was added daily to each animal's ration.

**Results.** All animals receiving 0.1 % of irradiated ergosterol in their diets lost weight rapidly and were dead after the lapse of 20 days and upwards. All animals receiving the smaller concentrations of irradiated ergosterol however grew normally and appeared to thrive during the same period. Nor could any ill effect be observed during the test period in any of the controls receiving the various concentrations of non-irradiated or of heated ergosterol (see Fig. 1).

The rats receiving the toxic concentration of 0.1 % of irradiated ergosterol were observed to lose their appetite early in the experiment, and this loss gradually increased. Diarrhoea set in generally at about the 5th day. Lesions on the hind paws superficially bearing some resemblance to pellagra, but of no marked severity, frequently attracted attention after a fortnight. The coats became greasy and very rough, and there was marked cachexia. The *post mortem* appearance was that of inanition, recalling therefore an effect of vitamin B deprivation. The pathological changes are still under examination and will form the subject of a later communication.

In Fig. 2 are shown the results of an experiment in which the above observations were repeated, using diets in which the ergosterol, irradiated and non-irradiated, had been incorporated three months previously; the daily food consumption was also noted approximately.

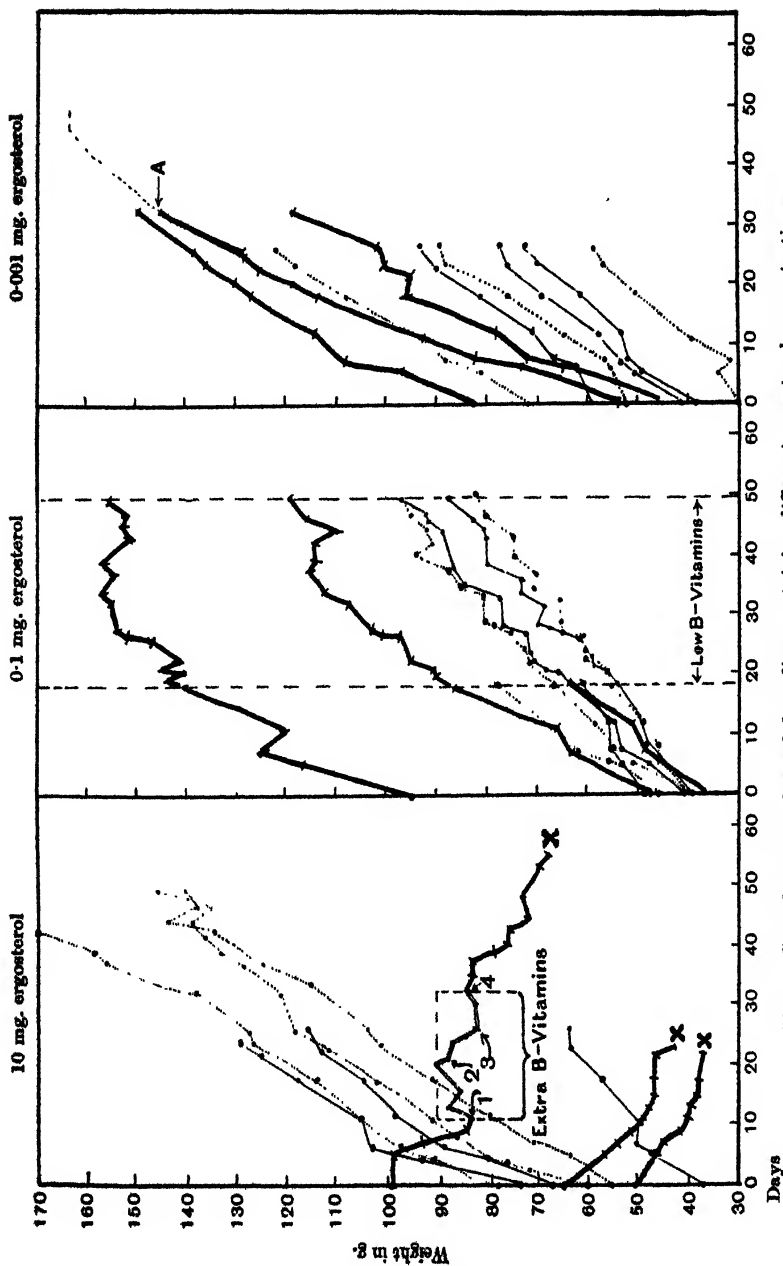
It is obvious, of course, that the ill-effects may quite conceivably have been caused by impurities in the irradiated ergosterol and not by the vitamin D itself, but since we have found that heated ergosterol has not the same deleterious effect it is clear that impurities which are common to the processes of heating and irradiation cannot be held responsible. Similar tests are now in progress on specimens of activated ergosterol in which the D factor has been destroyed by over-exposure to irradiation<sup>2</sup>. Among other aspects of the problem, we are investigating at present the following: intestinal reaction; the Ca and P absorption and blood levels; electro-cardiographic records.

## II. *Excessive irradiated ergosterol with increased B (and C) vitamin allowance.*

Efforts have been directed to determine whether the harmful effects of excess of irradiated ergosterol could be "balanced" by an increase of the vitamin B-complex. In one experiment, where the marmite allowance was irradiated ergosterol ("radiostol"). The possibility may be borne in mind of different methods of irradiation producing a different degree of toxicity in any by-products, apart from the clear evidence of true hypervitaminosis-D as revealed in excessive calcification, hypocalcaemia, hyperphosphataemia (Kreitman and Moll, Hess and Lewis; see Discussion, p. 1475).

<sup>1</sup> The extract is that used at the Cambridge Biochemical Laboratory: marmite is shaken with successive quantities of 85 % alcohol, the alcohol is removed by evaporation from the extract thus obtained, and the resulting fluid is filtered and made up with distilled water to a total volume equal to the weight of the marmite originally taken.

<sup>2</sup> We have found that a lethal dose of irradiated ergosterol becomes non-toxic concurrently with the destruction of vitamin D by over-irradiation.



Days

Fig. 1. Growth curves of rats fed on diets containing different ergosterol concentrations. Rapid loss of weight and death occurred with 10 mg. of irradiated ergosterol per 10 g. of diet. At the lower concentrations of 0.1 mg. and 0.001 mg. of irradiated ergosterol per 10 g. of diet, growth occurred over the same period. With non-irradiated and with heated ergosterol growth was supported at all three levels. (At the point "1" the marmite allowance was increased from 0.75 to 3.0 cc., at "2" 0.5 cc. of a wheat germ extract, and at "3" 10 cc. of orange juice were added, per diem. At "4" these additions were discontinued.)

==== Irradiated ergosterol, extra B vitamins  
 — Heated ergosterol.

..... Non-irradiated ergosterol.

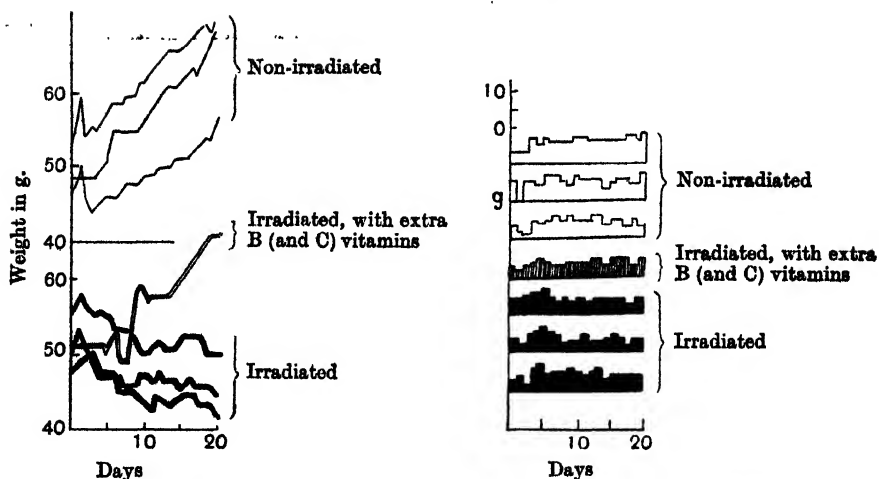


Fig. 2. Growth curves and food intakes. Diet contained 0.1 % ergosterol, incorporated 3 months previously.

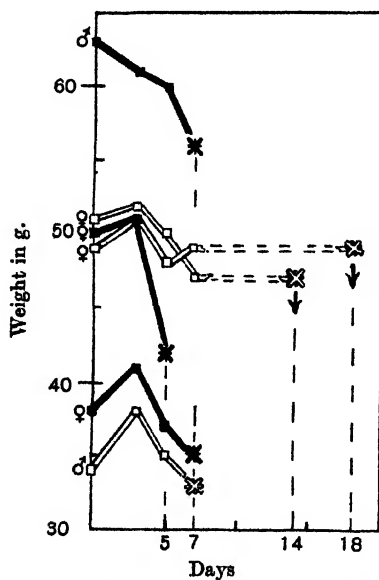


Fig. 3. Comparison of "D-hypervitaminosis," in young albino rats, on normal and fourfold vitamin B allowance.

(0.1 % irradiated ergosterol.)

— 0.75 cc. marmite extract.

--- 3.00 cc. marmite extract.

✕ ✕ Denote date of death.

increased to only four times the normal minimal level for good growth (viz. from 0.75 cc. of our extract to 3 cc.), a negative result was obtained. The rats (albinos in this instance) declined almost as rapidly and died about as soon as those whose marmite allowance remained at one-quarter the amount (Fig. 3). (An experimental difficulty is that rats on a normal diet will not thrive when given more than 3-4 cc. of our marmite extract, so that while the vitamin D has been increased 100,000 times above the minimal dose the vitamin B in the present case has not been increased by more than four times the minimum.)

In the opposite direction we can at present refer to two animals only, which when given still more vitamin B (fourfold marmite, plus wheat germ extract and orange juice) remained steady or gained in weight (see Figs. 1 and 2) while their mates, whose ration was not so balanced, declined rapidly, as mentioned above. Appetite improved, the animals remained apparently healthy, and their coats continued in good condition instead of being rough and greasy. Diarrhoea, however, was not averted. One animal on having the vitamin B allowance reduced to the normal level then began to lose weight and later died (see Fig. 1).

### III. *The apparent ability of the rat to discriminate between normal doses and toxic overdoses.*

An attempt was made to produce severe "hypervitaminosis" in rats by the administration of a single massive dose of vitamin D. Two rats were offered 5 g. of synthetic diet containing 0.25 g. of irradiated ergosterol but they refused it, consuming only a negligible quantity, while a litter mate readily consumed 5 g. of the same diet differing only in containing 0.25 g. of ergosterol in the non-irradiated form. The former animals lost weight rapidly from starvation and the experiment had to be abandoned; the latter grew normally for 18 days following (*A* in Fig. 1) and showed no ill effects, when the observations were concluded.

In a confirmatory experiment two rats were taken off a mixed dietary and offered the choice between synthetic diets containing excessive (1) non-irradiated, and (2) irradiated ergosterol, and also (3) no ergosterol. (The third named was only added after the lapse of a day when little of the first two had yet been eaten.) The ergosterol (0.025 g.) was added in chloroform solution to 5 g. of diet and the chloroform evaporated, the diet with no ergosterol being treated with chloroform in the same way. Allowance was made for the small amount of water lost from the ration during the time it was offered. As the figures below show, the non-irradiated ergosterol was eaten in preference to the irradiated. The experiment was repeated a second time with similar results.

	Food eaten (g.)			
	Rat No. 1	Rat No. 1. Repeat	Rat No. 2	Rat No. 2. Repeat
Containing irradiated ergosterol	0	0	0.3	0
Containing non-irradiated ergosterol	3.0	4.0 (all gone)	5.2	2.0 (all gone)
Containing no ergosterol	4.3	None offered	1.0	None offered



As in Section I, we have no evidence whether the effect be due specifically to the high concentration of vitamin or to some distasteful impurity. Considerations of expense almost preclude any large-scale repetition of the present observation, but it may be concluded that it is important to consider the question of loss of appetite or distaste for food in interpreting the results in the other sections<sup>1</sup>.

#### IV. *Vitamin B deprivation accompanied by increased vitamin D allowance.*

These experiments were planned in order to determine if one could discover any very simple relationship of "vitamin balance" in the sense that excess of one vitamin might be the more harmful in proportion to coexisting deficiency of the other, and *vice versa*.

*Procedure.* A concentration of 0.001 % of irradiated ergosterol was employed, *i.e.* one-hundredth of what may be called the lethal concentration (or one-hundredth of Kreitmair and Moll's toxic dose of 10 mg., assuming the animal to consume, as it does, approximately 10 g. of food). As a preliminary diet for 18 days, three rats received the complete ration containing 0.75 cc. of the marmite extract already described and 0.001 % of ergosterol, non-irradiated, irradiated and heated in the three cases. During this period somewhat subnormal growth occurred. Two rats in each of the three sets then received the diminished allowance of only 0.25 cc. of marmite extract, and one rat in each set no vitamin B.

*Result.* In the three animals deprived of all vitamin B, cessation of growth, loss of weight and finally death took place at much the same rate or time, irrespective of the amount of vitamin D in the diet (see Fig. 4). Rats receiving the restricted (one-third adequate) amount of vitamin B represented by 0.25 cc. of marmite extract showed no striking difference in their growth curves with variation in their vitamin D allowance (see Fig. 1, centre).

The rats receiving about 1000 times the adequate supply of vitamin D, it will be observed, succumbed no more rapidly to B-avitaminosis than is usual with rats receiving no increased vitamin D. Nor was one-hundredth of a toxic dose of irradiated ergosterol converted into a toxic dose when the vitamin B allowance was reduced to one-third. The experiment is of course not necessarily conclusive against the existence of a "vitamin balance", except in the special sense indicated.

#### IV. *Excessive cod-liver oil and retarded growth.*

Two carefully matched sets (*A* and *B*) of young albino rats were used, each set being divided into four groups. Similar synthetic diets (20 % caseinogen, 15 % fat, 40 % starch, 5 % salts) were fed to each set, but whereas set *A* had the 15 % fat supplied by a highly potent cod-liver oil, set *B* received the

<sup>1</sup> It may be added that these observations can be paralleled in another investigation, in the course of which one of us (L. J. H.) has found that an animal starved of one of the B vitamins is able to discriminate between diets containing a bare sufficiency and those devoid of it.

corresponding amount of inactive arachis oil together with a single drop of cod-liver oil per rat per day, to ensure a bare sufficiency of vitamins A and D. The groups in each set were then placed on graded allowances (1.6 %, 3.2 %, 6.4 %, 12.8 %) of our standard marmite extract, in order that the effect of the two oils might be compared at various levels of vitamin B intake.

*Results.* Invariably rats in set *A* (15 % cod-liver oil) recorded lower growth rates than carefully matched litter mates in set *B* (15 % arachis oil) (see Fig. 5). In Fig. 7, for convenience in presentation, the curves for each

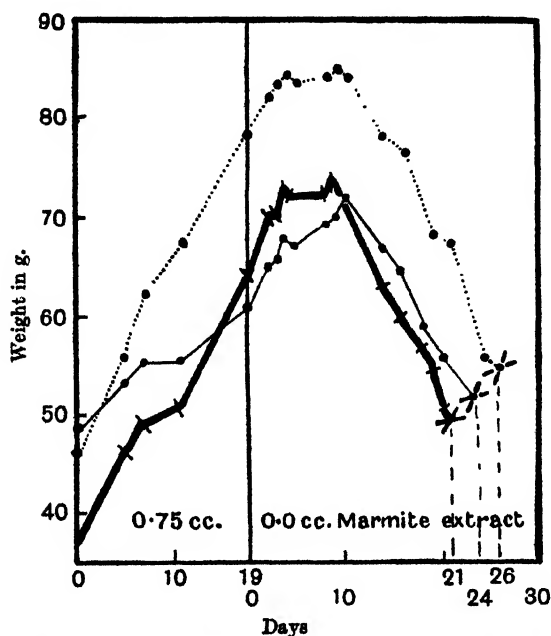


Fig. 4. B-avitaminosis with increased vitamin D allowance. The extra vitamin D provided did not appreciably retard the avitaminosis.

— Irradiated ergosterol, 0.1 mg. per 10 g. diet.  
 ... Non-irradiated " " "  
 — Heated " " "

group have been compounded and averaged. Differences were most marked at the intermediate marmite concentrations (3.2 and 6.4 %) adequate for maintenance but inadequate for rapid growth. Only small differences were observed at the highest (12.8 %) and lowest (1.6 %) marmite levels, the latter being inadequate for prolonged maintenance. The animals receiving cod-liver oil, except for marked abnormalities discussed under the next heading, remained in fair condition, though they could readily be distinguished from animals of the other groups by the roughness of their coats, which became distinctly yellowish in colour. The area around the genitals and anus was also found invariably to be stained a deep yellowish colour.

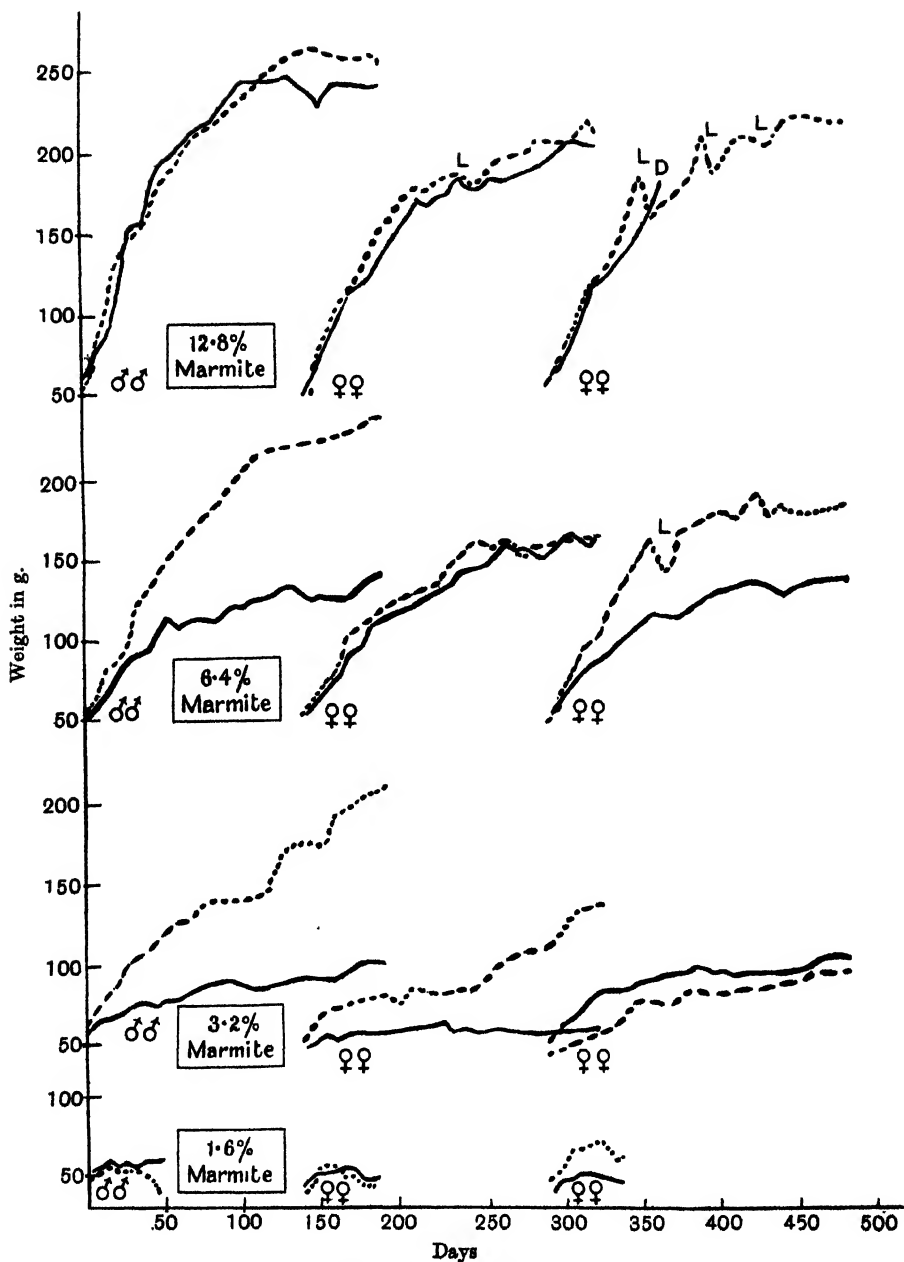


Fig. 5. Excessive cod-liver oil.  
 Continuous curves—Cod-liver oil diet.  
 Dotted curves—Arachis oil diet.

To confirm the above results the experiment was repeated at those concentrations of marmite (3.2 and 6.4 %) at which the greatest divergence had been observed. Exactly similar results were obtained (see Fig. 6).

#### V. Failure of pregnancy on excessive cod-liver oil.

In the preceding experiments sexes had remained together for several months, and as a result substantial support is afforded to the view that excess of cod-liver oil leads to failure of reproduction in rats. Since a detailed investigation was not made it is impossible to state whether failure in conception or resorption was usually the cause of sterility. The litters obtained were as follows.

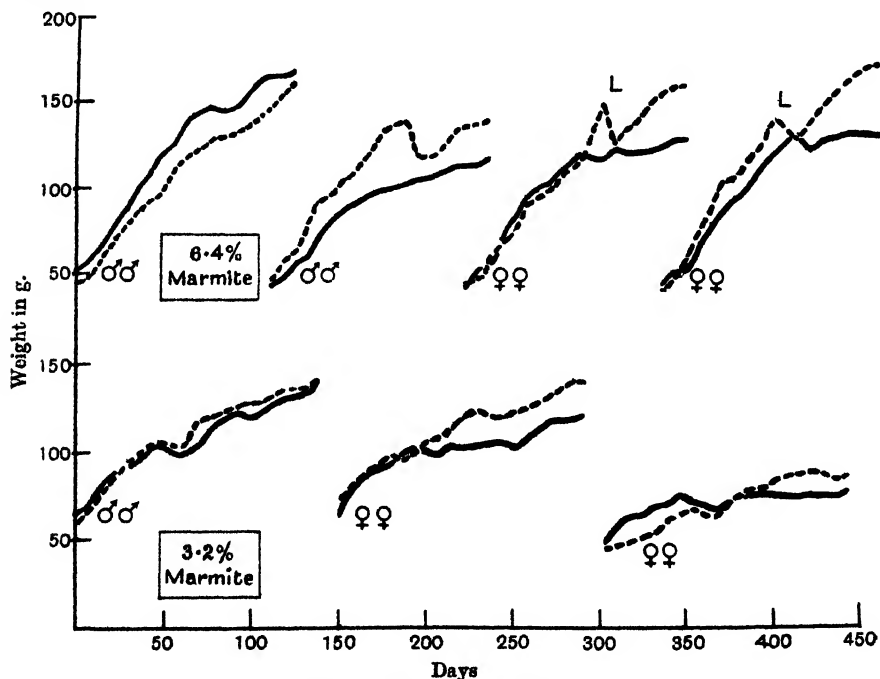


Fig. 6. Excessive cod-liver oil.  
Continuous curves—Cod-liver oil diet.  
Dotted curves—Arachis oil diet.

*Set A.* No litters were obtained in any group. One rat in the group receiving 12.8 % marmite became pregnant, but died during pregnancy. An autopsy revealed severe haemorrhage into the uterus. One developed foetus was found together with several small, apparently degenerated foetuses.

*Set B.* Seven litters were obtained in all, four from two rats receiving 12.8 % of marmite extract in their diet, three from four rats receiving 6.4 %. No litters were obtained from the groups receiving 3.2 % and 1.6 % of marmite extract. In no case were litters successfully reared, but they were often kept for periods up to 20 days.

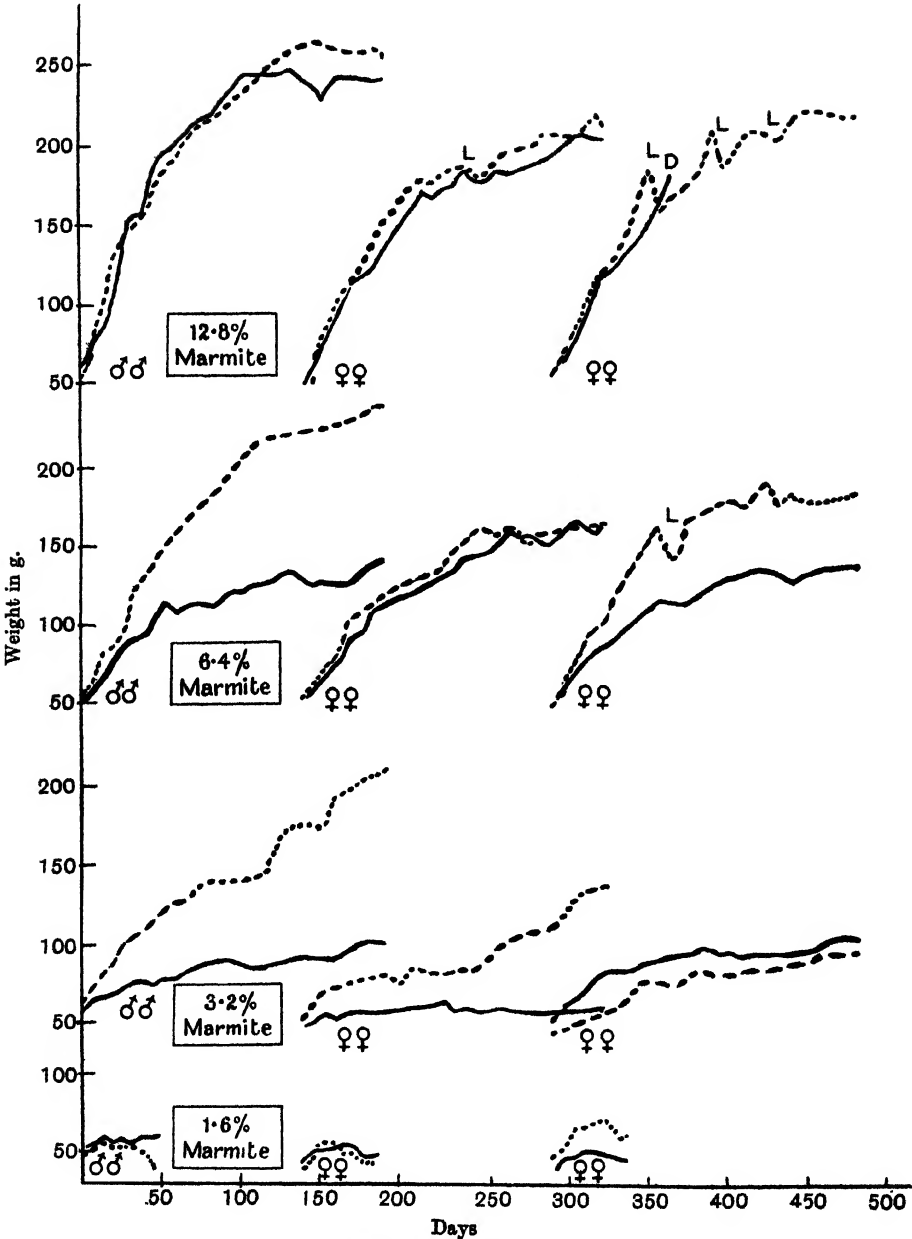


Fig. 5. Excessive cod-liver oil.  
Continuous curves—Cod-liver oil diet.  
Dotted curves—Arachis oil diet.

Vol. 22, p. 1468 one line from bottom:  
for 40 % starch read 60 % starch

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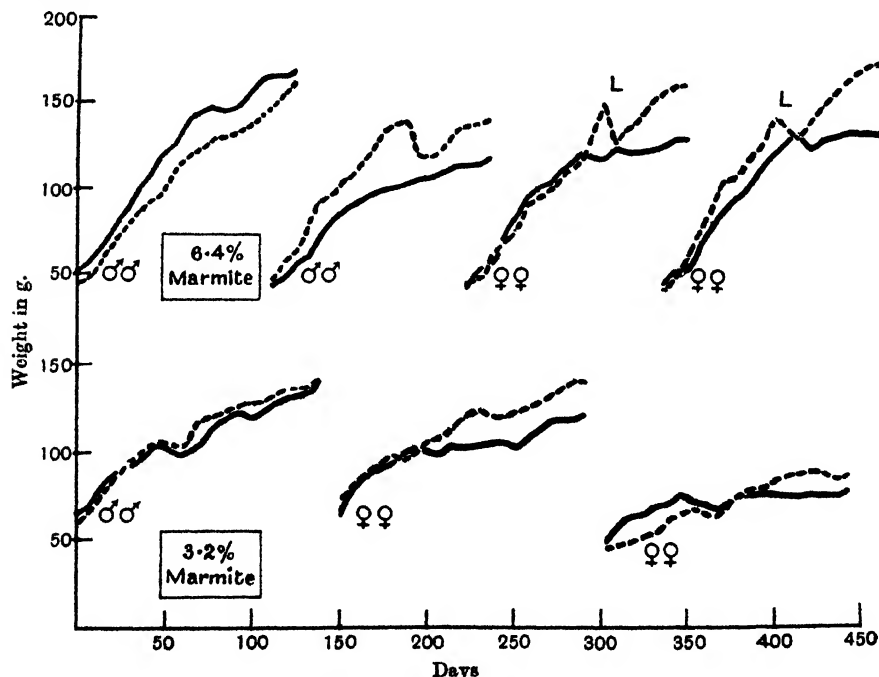


Fig. 6. Excessive cod-liver oil.  
Continuous curves—Cod-liver oil diet.  
Dotted curves—Arachis oil diet.

*Set A.* No litters were obtained in any group. One rat in the group receiving 12.8 % marmite became pregnant, but died during pregnancy. An autopsy revealed severe haemorrhage into the uterus. One developed foetus was found together with several small, apparently degenerated foetuses.

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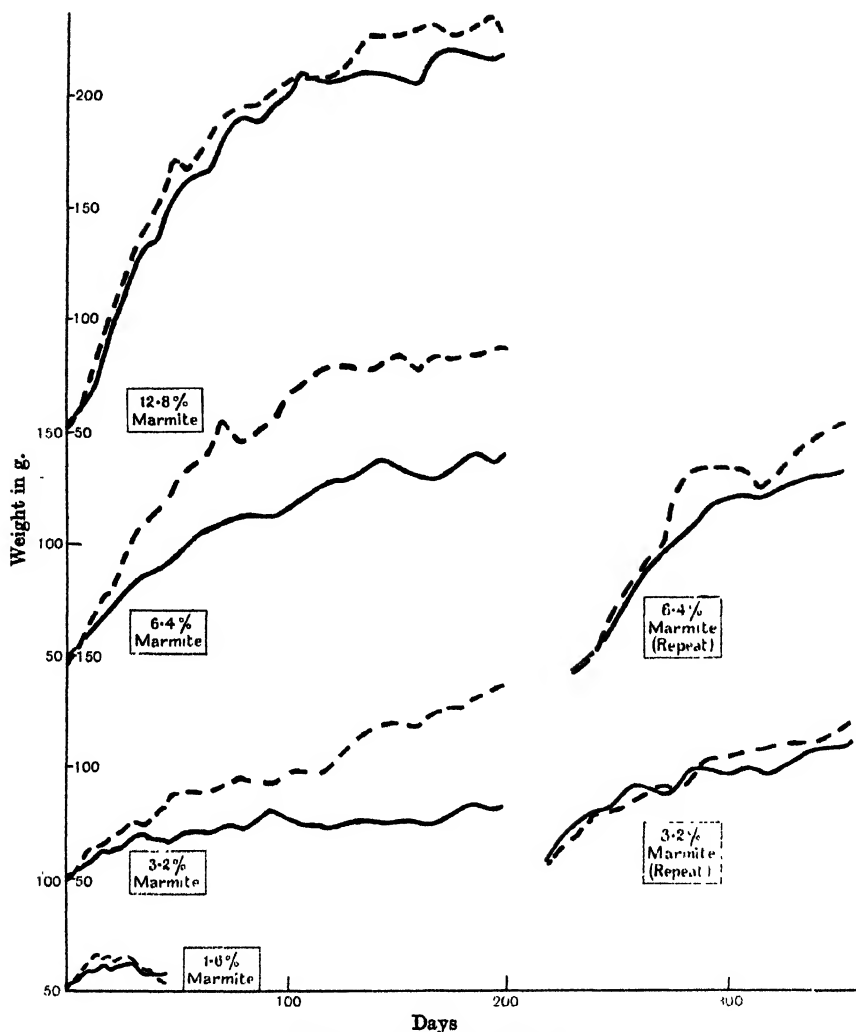


Fig. 7. Excessive cod-liver oil.

Average growth rates of groups.  
Continuous curves—Cod-liver oil diet.  
Dotted curves—Arachis oil diet.

#### VI. Excess of cod-liver oil concentrate with deprivation of B vitamins.

Two groups (*A* and *B*) of three young piebald rats each were used. Each received a standard basal diet (B.D.H.) without addition of marmite. For each rat in group *A* five drops of the unsaponifiable matter of cod-liver oil<sup>1</sup> were added, which were carefully stirred into the diet.

<sup>1</sup> Obtained through the courtesy of Messrs Lever Bros.

Both groups, through deprivation of the vitamin B complex, soon began to lose weight (Fig. 8), but in group *A* (receiving concentrate) such loss was much more rapid than in group *B*. After a few days a marked difference in appearance between the two groups could be observed. The rats in group *B*, though gradually weakening, were no more rough coated than was consistent with a short period of vitamin B deprivation. The rats in group *A*, on the other hand, began to lose hair around the mouth and paws, and skin lesions and bleeding soon developed. The condition superficially resembled severe pellagra<sup>1</sup> or the "egg-white" condition reported by Boas [1927].

In preliminary experiments attempts to alleviate the condition by the administration of marmite met with failure. Further experiments in this connection are now in progress.

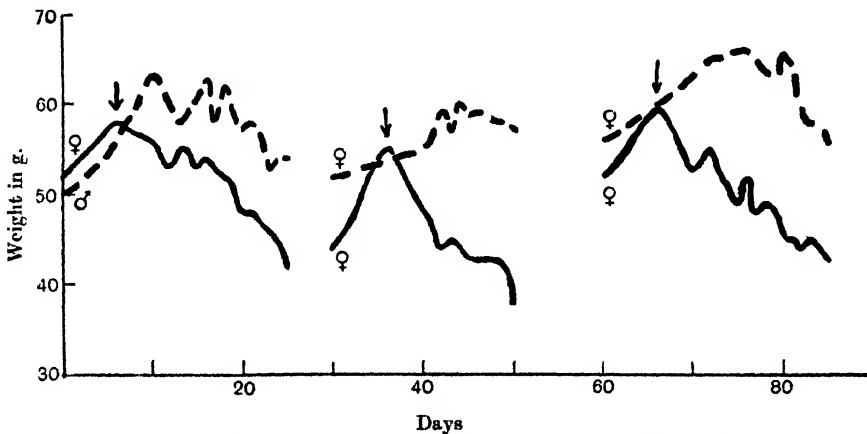


Fig. 8. Excess of cod-liver oil concentrate on vitamin B-free diet.

Continuous curves—With concentrate.

Dotted curves—Without concentrate.

↓ = Concentrate first administered.

#### DISCUSSION.

"*Hypervitaminosis.*" In support of the contention of Pfannenstiel and Kreitmair and Moll that vitamin D is injurious when consumed in large excess we have found that irradiated ergosterol, cod-liver oil and cod-liver oil concentrate are all more or less harmful in excess, the degree of toxicity in each case being roughly in line with the amount of vitamin D reputed to be present. Moreover, crystalline ergosterol, over-irradiated ergosterol and ergosterol resinised by heating have been found apparently harmless when consumed for the same periods at levels definitely lethal in the case of irra-

<sup>1</sup> Symptoms of vitamin B<sub>3</sub> deficiency are, of course, not observed in simple deprivation of the vitamin B complex, because the animal succumbs to the deficiency of vitamin B<sub>1</sub> before the effect of B<sub>3</sub> deficiency becomes apparent.



diated ergosterol—which suggests a close parallelism between the development of antirachitic and of toxic properties.

As a possible criticism it might be argued that the injurious effects could in all cases be attributed not to the vitamin but to other substances accompanying it which might have been either directly poisonous or else indirectly harmful by rendering the diets too distasteful to be eaten. To the question of direct poisoning no complete answer can be given, since, for the present, vitamin D cannot be administered free from impurities. To the question of indirect harm by starvation the answer may be given that the rats, although suffering considerable diminution in appetite on the introduction of the irradiated ergosterol or cod-liver oil concentrate into the diet, at least consumed sufficient amounts of the food to keep them alive for periods of about 20 days. Moreover, the possibility cannot yet be excluded of excess of vitamin D serving to unbalance the vitamin B allowance, and thereby rendering insufficient what would be normally an adequacy. In this case the most noticeable effect of any such virtual deficiency of vitamin B would indeed be a loss of appetite such as is observed. Again, rats receiving excess of irradiated ergosterol developed various symptoms, including diarrhoea, which could not be attributed to simple starvation.

Although we consider our results to have been consistent with the theory of hypervitaminosis it is not desired for the present to claim for them a too liberal interpretation. All that can be stated with certainty is that several materials commonly used as rich sources of vitamins (A and D, or D only) have been found to exert definitely harmful effects when administered to rats in amounts greatly in excess of the sufficient physiological level. Although a weight of evidence points to the toxic action of excess of vitamin D there is no necessity to assume that the ill effects observed were invariably attributable to that factor. Granted the toxicity of vitamin D excess, the question of vitamin A excess, for example, remains open. In cod-liver oil there are certainly many constituents of whose chemical nature and properties little is known. We allude to this because the simpler view is sometimes adopted that replacement of inert fats by cod-liver oil in a diet can be represented from the nutritional point of view simply as an increase in the A and D factors.

To obtain further evidence as to the degree of association of the toxic effects with vitamin D, *per se*, experiments are in progress to decide whether materials rich in the vitamin retain or lose such effects when the vitamin is destroyed, *e.g.* by prolonged over-irradiation. Further light is also being thrown upon this issue by investigations now proceeding on the effect of massive vitamin D intake upon calcium and phosphorus metabolism and on acid-base equilibrium in the gut.

“*Vitamin balance.*” Since rats receiving only the normal allowance of marmite (B vitamins) suffered from no marked symptoms of vitamin B deficiency when irradiated ergosterol was given at a rate 1000 times the minimal physiological

dose, it must be obvious that any possibility of simple arithmetical equivalence in balance between vitamin D and the B vitamins is out of the question. Pointing to the same conclusion we have the failure of this concentration of irradiated ergosterol to accelerate the decline which results from deprivation of the B vitamins (in which case the balance would be upset to the utmost degree).

Evidence as to the existence of a less quantitative form of balance, *e.g.* the ability of a relatively slightly increased vitamin B intake to modify the harmful effect of large excess of irradiated ergosterol is less clear-cut. Rats receiving "toxic" amounts of irradiated ergosterol (*i.e.* 100,000  $\times$  minimal effective dose) in a limited number of cases seemed to derive but slight benefit from a fourfold increase in the marmite allowance, but by the further introduction of wheat-germ extract and orange juice into the diet some favourable results have been obtained. These observations of course need extending.

In regard to the retardation of growth by unconcentrated cod-liver oil at various levels of marmite allowance, a study of the growth curves (Figs. 5, 6 and 7) might be considered to afford evidence of a balance between vitamins A (and D) and B—since in the presence of cod-liver oil larger amounts of marmite were required to produce any given growth rate than in its absence. It is not however our desire to advance this view, since the apparent balance can be more simply explained as a superimposition of the ill effects of large excess of cod-liver oil upon those incidental to partial vitamin B deprivation. Similarly, the fact, already alluded to, that certain of the symptoms of "D hypervitaminosis" were superficially similar to those of vitamin B deficiency may be no more than accidental.

Summarising the evidence we would suggest that, while the conception of a strictly quantitative balance cannot be tenable in any general sense, the possibility of large excess of one vitamin emphasising the effects of deficiency of another cannot as yet be ruled out.

*Practical applications.* In view of possible alarmist fears prejudicial to the use of the preparations (irradiated ergosterol and cod-liver oil concentrate) placed at our disposal by manufacturing houses we wish in conclusion to make clear that the doses required to produce toxic effects were at levels altogether outside those that are in practice used commercially or that could be employed in clinical practice.

In the first place, we have ourselves tested certain samples of margarine in which the vitamins A and D concentrate is employed and have satisfied ourselves both as to its vitamin value and the absence of any ill effects.

As to the clinical aspect<sup>1</sup>, roughly calculating our doses for a child of 28 pounds weight on a basis of relative body weight, we find that 2 g. of irradiated ergosterol, 20 g. of cod-liver oil concentrate and 300 g. of cod-liver oil would

<sup>1</sup> See Harris [1928].

have been administered daily. Toxic effects at such enormous dosages should not in any way discourage the rational use of the properly standardised materials<sup>1</sup> at the ascertainable correct physiological levels<sup>2</sup>.

#### SUMMARY.

(1) Young rats lost weight rapidly and died when receiving synthetic diets containing 0.1 % of an irradiated (but not non-irradiated, over-irradiated or heated) ergosterol (*i.e.* about 100,000 times a minimal protective dose). There was loss of appetite, ill condition of coats, etc., diarrhoea, and inanition.

(2) When the vitamin B (marmite) allowance was increased to only four times the normally adequate level no appreciable alleviation of these symptoms resulted. In the two cases where still further vitamin B (and C) (wheat-germ extract plus orange juice) was administered, loss of weight was prevented.

(3) Results due to "toxicity" are contrasted with those due to mere loss of appetite. Reference is made to the apparent ability of the rat to discriminate in its choice of diets. In a quantitative study rats were found to refuse food overloaded with irradiated ergosterol (5 %).

(4) Rats were found to have lower growth rates compared with litter mates, and to have rough coats, when cod-liver oil was substituted for 15 % of arachis oil (inactive) in a ration containing restricted allowances of vitamin B complex.

(5) In confirmation of Hartwell and of Sure, we observed that normal gestation always failed in rats receiving a diet containing 15 % of cod-liver oil.

(6) Rats receiving massive doses of vitamins A and D concentrate from cod-liver oil, in conjunction with a diet deficient in the vitamin B complex, developed loss of hair and severe skin lesions.

The feeding and care of the experimental animals have been in the experienced hands of Mr Alfred Ward.

<sup>1</sup> Different specimens of irradiated ergosterol, for example, probably vary considerably in vitamin D content.

<sup>2</sup> The report has recently appeared of Hess and Lewis [1928], who as a routine treated their rachitic patients with irradiated ergosterol at the admittedly high level of 2.5-5 mg. *per diem*, equivalent to about 35-70 teaspoonfuls of cod-liver oil. (This high dosage, however, has been widely advocated and appears to have some vogue.) Remarkable benefit resulted in every one of their large number of cases; there were, however, several instances of "eburnation" (hypercalciification), three cases of abnormally high blood-phosphorus, and several of hypercalcaemia, two of the latter showing clinical disturbances which were rectified when the irradiated ergosterol was omitted.

## REFERENCES.

- Agduhr (1926). *Acta Paediatrica*, 5, 319.  
Bezssonoff (1923). *Bull. Soc. Hyg. Aliment*, 11, 14.  
Boas (1927). *Biochem. J.* 21, 712.  
Drummond, Channon and Coward (1925). *Biochem. J.* 19, 1047.  
Euler and Widell (1925). *Z. physiol. Chem.* 144, 132.  
Funk (1922). *The vitamins* (Baltimore, 217).  
Grant (1926). *Dental Cosmos*, 68 (2), 899.  
Harris (1928). *Brit. Med. J.* ii, 964.  
Hartwell (1927). *Biochem. J.* 21, 1076.  
Havard and Hoyle (1928). *Biochem. J.* 22, 713.  
Hess and Lewis (1928). *J. Amer. Med. Assoc.* 91, 783.  
Höjer (1926). *Acta Paediatrica*, 6 (ii).  
Hopkins (1923). *Brit. Med. J.* ii, 693.  
Kreitmair and Moll (1928). *Münch. med. Woch.* 75, 637.  
Kroetz (1927). *Klin. Woch.* 6, 1171.  
Magliano (1925). *Riv. Biol.* 7, 619.  
Mouriquand and Michel (1922). *Compt. Rend. Soc. Biol.* 7, 1170.  
Pfannenstiel (1927). *Klin. Woch.* 6, 2310.  
Rosenheim and Webster (1927). *Biochem. J.* 21, 391.  
Sure (1927). *J. Biol. Chem.* 74, 45.  
Takahashi (1922). *J. Chem. Soc. Japan*, 43, 826.  
Takahashi and Kawakami (1923). *J. Chem. Soc. Japan*, 44, 580.  
Takahashi, Nakamiya, Kawakami and Kitasato (1925). *Scientific Papers Inst. Phys. Chem. Res. (Tokyo)*, 3, 81.

# CLXXXIII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS. PART IV.

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*(Received November 5th, 1928.)*

THE work described in Parts I, II and III [Butcher, Pentelow and Woodley, 1927, 1928] has now been concluded and the present paper includes the following experiments over periods of 24 hours:

- R. Lark.* Series XI. January 25-27, 1928. Table XXII.  
Series XII. February 16-17, 1928. Table XXIII. Fig. 19.  
Series XIII. March 1-2, 1928. Table XXIV. Fig. 20.  
Table XXV. Summary of R. Lark results. March 1927-March 1928.
- R. Itchen.* Series IX. January 17-18, 1928. Table XXVI. Fig. 21.  
Series X. February 21-22, 1928. Table XXVII. Fig. 22.  
Series XI. March 20-21, 1928. Table XXVIII. Fig. 23.  
Series XII. May 3-4, 1928. Table XXIX.

## *Variation in dissolved oxygen.*

*River Lark.* A few observations were made on January 25th-27th, 1928, when the effects of the pollution by the effluents from the beet-sugar factory were less marked than in November 1927, and it will be seen that the diurnal variation effect is still evident in this polluted water.

The experiment of February 16th-17th was made when the effects of the winter pollution were disappearing, sewage fungus was still abundant, and the diatoms were first beginning to appear. The range of variation of oxygen content was smaller than at any other time of the year, viz. 66-77 %, indicating the scarcity of actively assimilating plants, and this was confirmed by the absence of variation in  $p_H$  value. The figures obtained in March 1928 indicate the commencement of that rapid increase of diatoms which occurs every spring.

**Table XXII. *River Lark. January 25th-27th, 1928. Series XI.***

Sunset January 25th, 4.36 p.m.  
Sunrise „ 26th, 7.48 a.m.  
Sunset „ 26th, 4.37 p.m.

Sunset February 16th, 5.15 p.m.  
Sunrise „ 17th, 7.13 a.m.  
Sunset „ 17th, 5.17 p.m.

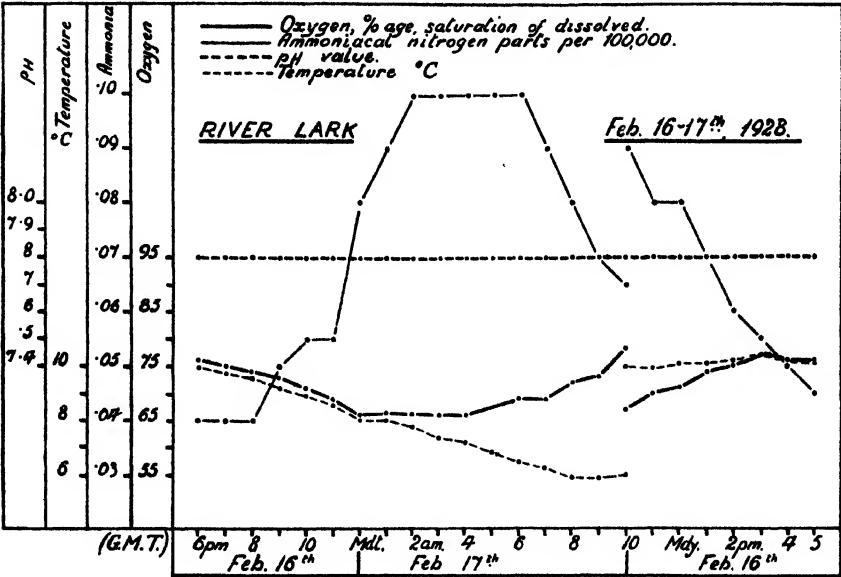


Fig. 19.

Table XXIV. *River Lark. March 1st-2nd, 1928. Series XIII.*

Time	Temp. ° C.	Dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	pH value	Remarks
		Parts per 100,000	% satu- ration			
10 a.m.	7.6	0.791	65	0.13	7.5	Cloudy. Drizzle
11 "	7.7	0.866	72	0.11	7.7	" "
Midday	7.9	0.979	82.5	0.05-0.06	7.7-7.8	" Fine
1 p.m.	8.2	1.031	87	0.05	7.8	" "
2 "	8.4	1.149	97	0.04	7.9	" "
3 "	8.6	1.189	100	0.04	7.9-8.0	" "
4 "	8.7	1.187	100	0.04	7.9-8.0	" "
5 "	8.8	1.161	99	0.03	7.9-8.0	" "
6 "	8.8	1.036	88	0.02-0.03	7.9	Dusk. Cloudy. Drizzle
7 "	9.0	0.978	84	0.02-0.03	7.8-7.9	Dark. Cloudy. Fine
8 "	9.0	0.927	79	0.05	7.8-7.9	Cloudy. Fine
9 "	8.9	0.876	75	0.05	7.7-7.8	" "
10 "	8.8	0.846	72	0.05-0.06	7.7-7.8	" "
11 "	8.7	0.777	66	0.10	7.7	Mill stopped. Cloudy.
						Fine
Midnight	8.7	0.743	63	0.11	7.7	Cloudy. Fine
1 a.m.	8.7	0.714	61	0.11	7.6	" "
2 "	8.7	0.698	59	0.11	7.6	" "
3 "	8.7	0.653	55	0.13	7.5-7.6	" "
4 "	8.7	0.600	51	0.13	7.5-7.6	" Raining
5 "	8.7	0.632	54	0.13	7.5-7.6	" "
6 "	8.7	0.653	55	0.13	7.5-7.6	Dawn. Cloudy. Raining
7 "	8.7	0.659	55	0.16	7.5-7.6	Cloudy. Raining
8 "	8.7	0.663	56	0.16	7.5-7.6	" "
9 "	8.8	0.688	59	0.13	7.5-7.6	Sun breaking
10 "	8.9	0.713	61	0.11	7.5-7.6	Dull
11 "	9.1	0.812	69	0.11	7.6	Fine
1 p.m.	9.7	1.151	103	—	—	"

Sunset March 1st, 5.39 p.m.  
Sunrise " 2nd, 6.45 a.m.  
Sunset " 2nd, 5.41 p.m.

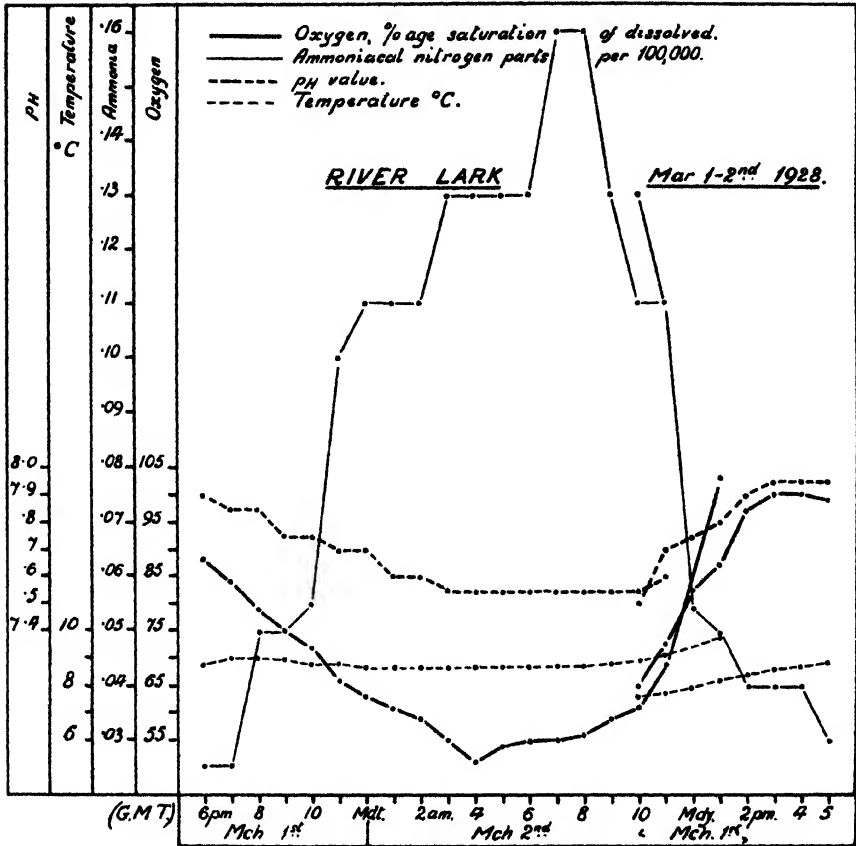


Fig. 20.

## FACTORS AFFECTING THE DISSOLVED OXYGEN CONTENT OF RIVER WATERS.

The main factors governing the dissolved oxygen content of a river water may be summarised as follows.

1. The time of day in its connection with the processes of photosynthesis and respiration.
2. The season of the year, with which varies:
  - (a) the quantity and class of plants and animals present,
  - (b) the length of the periods of light and darkness.
3. The prevailing actinic conditions.
4. The physical characters of the river.
5. Temperature variations.
6. The solution of oxygen from the atmosphere.
7. The degree and nature of any pollution.



Table XXV.

Date	% saturation of dissolved oxygen		pH value		Ammoniacal nitrogen		Temperature (° C.)		Weather	Sunrise a.m.	Plants
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum			
1927											
Mar. 30-31	3 p.m. 167	1-7 a.m. 65	2-7 p.m. 7.9-8.0	2-9 a.m. 7.6-7.7	2-5 a.m. 0.16	3-6 p.m. 0.01	3-4 p.m. 10.0	5 a.m. 6.75	Bright	5.40	Diatoms abundant, submerged and littoral plants just appearing
Apr. 27-28	3-4 p.m. 178	3-6 a.m. 57	2-6 p.m. 8.0	4-7 a.m. 7.5-7.6	3-9 a.m. 0.16	4-11 p.m. 0.02	6 p.m. 11.5	5-7 a.m. 7.0	Sunny	4.40	Submerged and littoral plants 1 ft. high. Diatoms abundant. Green algae appearing
May 18-19	4-5 p.m. 181	5-7 a.m. 36	4-8 p.m. 8.0	3-7 a.m. 7.5	6 a.m.-mdy. 0.2	6-10 p.m. 0.01-0.015	5 p.m. 15.4	7-8 a.m. 10.8	Sunny	4.5	Submerged plants at height of growth. Diatoms on decrease. Green algae dominant
June 28-29	3-4 p.m. 111	6 a.m. 37	5-9 p.m. 7.9-8.0	5-8 a.m. 7.4-7.5	6-10 a.m. 0.18	6 p.m.-mdt. 0.01	3 p.m. 14.5	8-9 a.m. 12.7	Dull. Rain	3.40	Submerged plants at height of growth. Cut June 22. Few diatoms
June 30	5-7 p.m. 110	—	—	—	—	—	6 p.m. 16.2	—	Afternoon brighter than on June 29		
Aug. 4-5	4-5 p.m. 124	7 a.m. 42	2-7 p.m. 8.0-8.2	5-8 a.m. 7.5	1-4 p.m. 0.015-0.02	7 p.m.-10 a.m. 0.01	5 p.m. 18.4	7 a.m. 15.7	Sunny	4.20	Submerged plants at height of growth. Some green algae
Sept. 7-8	4-5 p.m. 71	5-8 a.m. 39	4-6 p.m. 8.0	6-8 a.m. 7.4-7.5	2-5 p.m. 0.15	Mdt.-10 a.m. 0.01	2-5 p.m. 16.0	7 a.m. 13.1	Cloudy. Some sun	5.20	Weed-cutting
Sept. 22-23	5 p.m. 70	5-8 a.m. 26	5-7 p.m. 7.8	7-8 a.m. 7.5	9 a.m.-2 p.m. 0.05-0.06	7 p.m.-4 a.m. 0.01	1 p.m. 15.4	8 a.m. 11.95	Dull. Rain	5.50	
Oct. 12-13	3-4 p.m. 49	7-9 a.m. 27	2-4 p.m. 7.8	5-7 a.m. 7.4-7.5	8 a.m.-1 p.m. 0.11	7 p.m.-5 a.m. 0.01	1-11 p.m. 10.6	8 a.m. 9.9	Dull	6.20	
Nov. 17-18	2-3 p.m. 10	2 a.m.-mdy. 2	3 p.m. 7.6	5-7 a.m. 7.4-7.5	4 a.m.-mdy. 0.16	9-10 p.m. 0.025	1 p.m. 8.2	11 p.m. 7.2	Dull	7.20	Pollution
1928											
Feb. 16-17	3 p.m. 77	Mdt.-4 a.m. 66	10 a.m.-10 a.m. 7.8	10 a.m.-10 a.m. 7.8	2-6 a.m. 0.1	6-8 p.m. 0.04	3 p.m. 10.4	8-9 a.m. 5.9	Dull	7.15	Sewage fungus abundant. Plants few
Mar. 1-2	3-4 p.m. 100	4 a.m. 51	3-5 p.m. 7.9-8.0	3-10 a.m. 7.5-7.6	7-8 a.m. 0.16	6-7 p.m. 0.02-0.03	1 p.m. 9.7	11 p.m.-8 a.m. 8.7	Cloudy	6.45	Diatoms becoming abundant

Table XXVI. *River Itchen. January 17th-18th, 1928. Series IX.*

Time	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH value	Remarks
		Parts per 100,000	% satura- tion			
9 a.m.	5.4	1.051	82	0.005	7.7	Misty. River high and clear
10 "	5.3	1.095	86	0.005	7.7	Misty
11 "	5.1	1.140	88	0.005	7.8	"
Midday	5.3	1.174	90	0.005	7.8	Sunshine. Mist clearing
1 p.m.	6.0	1.184	94	0.005	7.8	"
2 "	6.7	1.201	97	0.005	7.8	"
3 "	7.0	1.172	95	0.005	7.8	"
4 "	7.1	1.138	92	0.005	7.7	Sun setting
5 "	6.8	1.100	89	0.005	7.7	Dusk. Clear
6 "	6.2	1.083	85	0.005	7.6	Dark. "
7 "	5.8	1.053	83	0.005	7.6	Ground mist
8 "	5.6	1.049	83	0.005	7.6	"
9 "	5.3	1.041	81	0.005	7.6	"
10 "	5.1	1.054	82	0.005	7.6	Clear "
11 "	4.9	1.046	81	0.005	7.6	"
Midnight	4.7	1.065	82	0.005	7.6	"
1 a.m.	4.5	1.058	81	0.005	7.6	"
2 "	4.3	1.061	81	0.005	7.6	"
3 "	4.3	1.054	80	0.005	7.5-7.6	Becoming cloudy
4 "	4.3	1.071	82	0.005	7.5-7.6	Cloudy
5 "	4.4	1.060	81	0.005	7.5-7.6	Very overcast
6 "	4.5	1.054	81	0.005	7.5-7.6	Drizzle
7 "	4.9	1.054	82	0.005	7.5-7.6	Raining. Dawn
8 "	5.0	1.047	81	0.005	7.5-7.6	Dull. Fine. Daylight
9 "	5.0	1.043	81	0.005	7.5-7.6	" "

Sunrise January 17th, 7.59 a.m.  
 Sunset " 17th, 4.21 p.m.  
 Sunrise " 18th, 7.58 a.m.

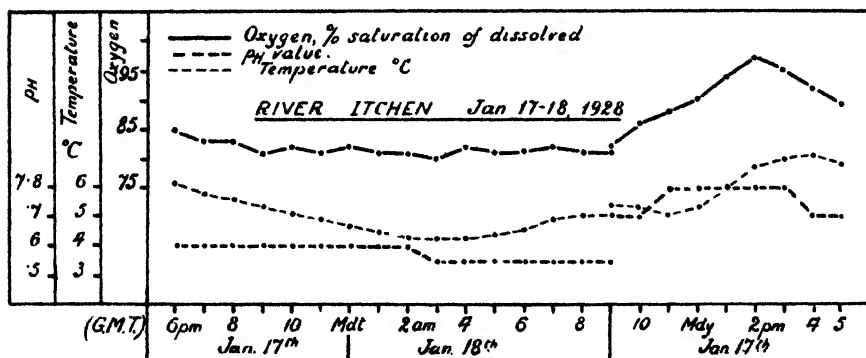


Fig. 21.

1. *Diurnal variation.*

The oxygen content varies diurnally, reaching its maximum value just after midday and having a period of minimum value during the night. The photosynthetic production of oxygen during the day and its continuous absorption by respiration and by chemical oxidation of organic matter throughout day and night result in a maximum concentration of dissolved oxygen a few hours after midday and a minimum content during the night, when photosynthesis has ceased.

Table XXVII. *River Itchen. February 21st-22nd, 1928. Series X.*

Time	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH value	Remarks
		Parts per 100,000	% satura- tion			
8 a.m.	5.3	1.006	79	0.005	7.7-7.8	Sunshine. Ground mist
9 "	5.5	1.084	85	0.005	7.7-7.8	"
10 "	5.9	1.137	90	0.005	7.8	"
11 "	6.5	1.173	94	0.005	7.8-7.9	"
Midday	7.1	1.222	99	0.005	7.9	"
1 p.m.	8.0	1.220	102	0.005	7.9	"
2 "	8.5	1.220	103	0.005	7.9	"
3 "	9.0	1.209	103	0.005	7.9	"
4 "	9.1	1.182	101	0.005	7.9	"
5 "	9.0	1.118	95	0.005	7.9	" Sun low
6 "	8.4	1.067	90	0.005	7.8	Dusk
7 "	7.8	1.033	85	0.005	7.6	Dark. Clear
8 "	7.2	0.990	81	0.005	7.6	Ground mist
9 "	6.8	0.991	80	0.005	7.6	"
10 "	6.2	0.981	78	0.005	7.6	"
11 "	6.0	0.978	78	0.005	7.6	"
Midnight	5.7	0.988	78	0.005	7.6	"
1 a.m.	5.4	0.978	77	0.005	7.5-7.6	"
2 "	5.1	0.990	77	0.005	7.5-7.6	"
3 "	5.0	0.990	77	0.005	7.5-7.6	"
4 "	5.0	1.003	78	0.005	7.5-7.6	"
5 "	4.9	0.994	77	0.005	7.5-7.6	"
6 "	4.9	1.011	78	0.005	7.5-7.6	Sky just lightening
7 "	4.8	1.002	77	0.005	7.5-7.6	Daylight
8 "	4.6	1.024	79	0.005	7.5-7.6	Sunshine

Sunrise February 21st, 7.5 a.m.

Sunset " 21st, 5.24 p.m.

Sunrise " 22nd, 7.3 a.m.

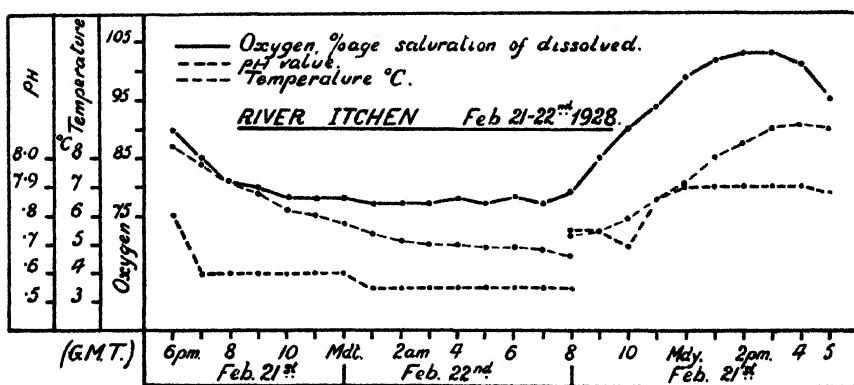


Fig. 22.

## 2. Season of the year.

## (a) Quantity and class of plants present.

1. *Diatoms*. A study of the maxima clearly shows that from March to May the water becomes supersaturated to a far greater extent than at any other time of the year. This period exactly coincides with the period of greatest growth and development of the diatoms (Part II), and there seems no doubt that it is these micro-organisms that are capable of production of oxygen so

Table XXVIII. *River Itchen. March 20th-21st, 1928. Series XI.*

Time	Temp. ° C.	Dissolved oxygen		Am- moniacal nitrogen. Parts per 100,000	pH value	Remarks
		Parts per 100,000	% satura- tion			
8 a.m.	7.9	1.040	87	0.005	7.8-7.9	Sunshine. Some clouds. Windy
9 "	8.0	1.160	97	0.01-0.015	7.8-7.9	" "
10 "	8.5	1.231	104	0.005	7.9	" "
11 "	9.4	1.237	107	0.005	7.9-8.0	Cloudy. Windy
Midday	9.6	1.255	108	0.005	7.9-8.0	" "
1 p.m.	9.7	1.226	106	0.005	7.9-8.0	" "
2 "	9.5	1.201	104	0.005	7.9-8.0	" "
3 "	9.2	1.151	99	0.005	7.9-8.0	" "
4 "	9.1	1.143	98	0.005	7.9-8.0	" "
5 "	9.1	1.078	92	0.005	7.9-8.0	" "
6 "	9.0	1.050	90	0.005	7.9	" Less wind
7 "	8.9	0.985	84	0.005	7.8-7.9	Raining slightly
8 "	8.8	0.955	81	0.005	7.8	" Dusk
9 "	8.5	0.925	78	0.005	7.8	Cloudy. Fine. Dark
10 "	8.3	0.931	78	0.005	7.7-7.8	Raining slightly. Windy
11 "	8.2	0.936	78	0.005	7.7-7.8	Raining. Windy
Midnight	8.1	0.914	76	0.005	7.7-7.8	" "
1 a.m.	8.0	0.930	77.5	0.005	7.7	" "
2 "	7.9	0.925	77	0.005	7.6	" "
3 "	7.6	0.923	76	0.005	7.6	" "
4 "	7.3	0.931	76	0.005	7.6	" "
5 "	7.2	0.938	77	0.005	7.6	" Sky just lightening
6 "	7.1	0.923	75	0.005	7.6	" Daylight
7 "	7.0	0.931	76	0.005	7.6-7.7	" Less wind
8 "	7.0	0.945	77	0.005	7.7	" "

Sunset March 20th, 6.11 p.m.

Sunrise " 21st, 6.3 a.m.

Sunset " 21st, 6.13 p.m.

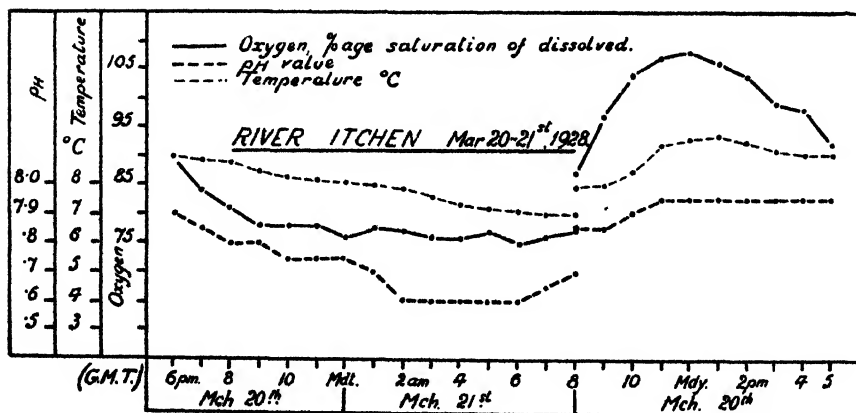


Fig. 23.

as to supersaturate the water to a very high figure. It is not that they necessarily produce more oxygen than any other plants, but, because of their small size, the size of the oxygen bubbles or particles must also be so small as to remain in solution in the water if not subject to violent agitation. Bubbles of oxygen evolved from flowering plants collect in the stomata and intercellular

spaces, where they enlarge and can be seen rising into the air, especially when the stem of a water-plant is broken.

The volume of diatoms produced in the Lark is considerably greater than in the Itchen and it is not the free floating organisms that are important but those growing on the river bed, for the latter are in numbers 10,000 times greater than the former.

Table XXIX. *River Itchen. May 3rd-4th, 1928. Series XII.*

Time	Temp. ° C.	Dissolved oxygen		Remarks
		Parts per 100,000	% saturation	
4 p.m.	14.1	1.119	107	Slightly overcast
9 "	12.5	0.590	54	
4 a.m.	10.8	0.556	49	Dawn
9 "	10.9	1.119	100	Bright and sunny
11 "	13.4	1.293	122	"
2 p.m.	15.4	1.362	129	"
4 "	15.9	1.261	125	"

Sunset May 3rd, 7.25 p.m.

Sunrise May 4th, 4.27 a.m.

What applies to diatoms should apply with equal force to other micro-organisms, *e.g.* *Protococcales* and *Schizophyceae*, but the volume of these is very small in the rivers under observation, and therefore insufficient to make any marked change in the oxygenation of the water. The best illustration of the work of the diatoms is shown in the results of February 16th and March 1st. It was not until the diatoms were present in quantity in March that the dissolved oxygen reached a high maximum.

2. *The flowering plants.* After the diatoms have decreased in volume almost to disappearance there remain the larger plants to oxygenate the water. Their importance is best shown by a comparison of the ecology of the two streams. In the River Lark the plants die down in the autumn and it is not till June that they are present in appreciable quantity, after which their volume increases very rapidly. It will be seen that when the flowering plants are present in greatest quantities the diurnal maximum of oxygen saturation is only 124 %, while in September after extensive weed-cutting had taken place the maximum oxygen figure is still lower at 70 %.

On the Itchen the flowering plants are in much smaller volume and the decrease from summer to winter is also small, the effect of which is shown in the constancy of the maximum oxygen values throughout the year with the exception of the "diatom" period.

The following summary of the results obtained on the River Lark indicates the influence of this seasonal variation in plant life upon the diurnal variation of dissolved oxygen in river water.

Date	Plant life	% saturation of dissolved oxygen	
		Maximum	Minimum
March 1927	Diatoms abundant. Submerged plants just appearing	160	65
April 1927	Diatoms abundant. Green algae appearing. Submerged plants 1 foot in height	180	60
May 1927	Diatoms decreasing. Green algae dominant. Large green plants growing vigorously	180	40
June 1927	Few diatoms. Submerged plants at height of growth. (Some weed-cutting had taken place)	110	40
August 1927	Submerged plants at height of growth. Some green algae	120	40
September 1927	After heavy weed-cutting	70	30
October 1927	Pollution by effluents from beet-sugar factory	(50)	(30)
November 1927	Pollution by effluents from beet-sugar factory	(10)	(2)
February 1928	Plants few. Sewage fungus abundant. Diatoms just beginning to appear, after cessation of pollution	77	66
March 1928	Diatoms becoming abundant	103	51

(b) *The effect of the seasonal variation of the duration of the periods of light and dark.*

This effect is seen in the differences in the lengths of the periods of minimum of oxygenation during the nights. During the summer, when the nights are short, the period of minimum of oxygen is correspondingly short, while in winter, when the nights are long, the period of minimum is extended over several hours.

### 3. *Prevailing actinic conditions.*

The effect of the variations in prevailing actinic conditions upon the periods and values of maximum and minimum oxygenation is small compared with that of the seasonal variation in plant life, and has been fully discussed in Part III.

### 4. *Type of stream.*

The depth of the stream, its speed of flow, and the nature of the bed, whether muddy or stony, have a considerable bearing upon the extent of the diurnal variation of dissolved oxygen. In general, the normal equilibrium between the oxygen of the atmosphere and that in solution in the river water is displaced by the effects of photosynthesis and respiration, and this displacement is more marked in the slower moving and deeper Lark than in the more turbulent and shallow Itchen, with the result that the range of diurnal variation of dissolved oxygen in the Lark is much greater than in the Itchen.

### 5. *Temperature variations.*

A study of the oxygen figures for June to October on the River Itchen (Series III to VI) shows that, whereas the maximum oxygen content of the river water expressed in parts per 100,000 remains constant, some variation is shown in the values expressed as percentage saturation of dissolved oxygen;

it will also be seen that the curve of the maximum values in percentage saturation follows the mean water temperature curve. This would indicate that the equilibrium between the oxygen of the atmosphere and that in solution is not maintained during a rise or fall in temperature; *e.g.* after a fall in temperature, oxygen is not immediately taken up from the air to maintain the original degree of saturation.

*Example. River Itchen. November 10th–11th, 1927.*

	Temp. ° C.	Dissolved oxygen	
		Pts/100,000	% saturation
10 p.m.	5.1	1.056	82
Midnight	4.7	1.056	81
6 a.m.	4.2	1.056	80

The ease with which this equilibrium may be established by solution of oxygen from the air is dependent to a certain extent upon the original oxygen saturation of the water, since the rate of solution of a gas in a liquid varies directly as the degree of unsaturation of the liquid, and consequently the temperature lag discussed in the previous paragraph is more evident in the region of saturation than in very subsaturated solutions. In general, this temperature effect is hidden by the larger and more rapid changes due to photosynthesis, respiration and pollution.

#### 6. *Oxygen absorption from the atmosphere.*

Throughout these experiments Fox's tables [1907] have been used for the conversion of parts per 100,000 to percentage saturation of dissolved oxygen, but it is questionable as to whether these theoretical values may be strictly applied to river waters, in view of the temperature lag discussed above, and since their use assumes that the solubility of oxygen in these river waters is the same as in distilled water.

#### 7. *The degree and nature of any pollution.*

A striking example of the overshadowing of nature's processes of river water aeration by the effects of industrial pollution is afforded by a study of the oxygen curves on the River Lark for October, November and January, 1927, when the river was practically deoxygenated throughout the day by the effluents from the beet-sugar factory 7 miles above the point of sampling.

#### $p_H$ VALUE OF RIVER WATERS.

Throughout the year the  $p_H$  curves were very similar in structure to those of saturation of oxygen, and showed the same effects of diurnal variation, seasonal variation of plant life and of the duration of night and day, indicating that the concentration of carbon dioxide is roughly inversely proportional to that of oxygen.

*Time of day.* The  $p_H$  value of the river water varied diurnally, having a maximum value during the afternoon due to the removal of carbon dioxide

during the photosynthetic production of oxygen, and having a period of minimum during the night, when photosynthesis ceases and carbon dioxide is still being liberated by the respiration of plants and animals.

*Season of year.* The values and periods of the diurnal variations in  $p_H$  value are affected considerably by the quantity and type of plant life present, depending upon the season of the year. The greatest range in  $p_H$  variation was obtained during the summer months when the large green plants were at the height of their growth, while on the River Lark in February 1928, when the plants were very few, no diurnal variation in  $p_H$  value was observed.

The periods of the maxima and minima are dependent upon the duration of the periods of light and dark, according to the season of the year, in a manner corresponding to the oxygen curves.

#### DIURNAL AND SEASONAL VARIATION IN AMMONIACAL NITROGEN.

The diurnal variation of ammoniacal nitrogen has been discussed in Part III, with the general conclusion that the nitrogen content varies in a sense opposite to that of oxygen.

On the Itchen no diurnal or seasonal variation was observed, while on the polluted River Lark considerable variations were observed throughout the year, although the data obtained were not sufficient to establish definite relationships between the nitrogen variation and the factors affecting it.

In conclusion the authors wish again to thank Sir R. Robertson and the Ministry of Agriculture and Fisheries for permission to publish these results, and Mr Stokes of the Fisheries Laboratory, Lowestoft, who is responsible for the graphs in all four parts.

#### REFERENCES.

- Butcher, Pentelow and Woodley (1927). *Biochem. J.* **21**, 945, 1423.  
——— (1928). *Biochem. J.* **22**, 1035.  
Fox (1907). *Conseil int. pour l'exp. de la mer*, **41**.



# CLXXXIV. ON OSCILLATIONS OF BLOOD-SUGAR VALUES WITHIN BRIEF PERIODS, AND THE BLOOD-SUGAR CURVE ON UNIFORM INGESTION OF GLUCOSE.

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*(Received September 3rd, 1928.)*

In late years a number of investigations on the blood-sugar have demonstrated not only that the daily fasting blood-sugar has no constant value, but also that, if instead of one single determination being made, the fasting blood-sugar is determined repeatedly through a certain period—with blood withdrawn at intervals of  $\frac{1}{4}$  to  $\frac{1}{2}$  hour—the blood-sugar curve obtained will show wide and long, more or less regular waves, which are supposed to be due to “sugar waves” issuing from the liver. Undoubtedly these waves are one of the reasons why the fasting blood-sugar value is found to change from day to day.

In addition to these rather regular curve waves Hansen [1923] has reported other oscillations in the blood-sugar, which were found on determining the blood-sugar at very frequent intervals for some time. Hansen found these oscillations, as distinct from the regular curve waves,

- (1) in normal individuals during fasting;
- (2) in diabetics during fasting, apart from the well-known steady fall in blood-sugar which is usually associated with fasting in hyperglycaemia;
- (3) after ingestion of carbohydrate, as the rise in blood-sugar does not proceed in a smooth curve but in connection with oscillations of the main grade. These oscillations are particularly brisk and relatively large when the blood-sugar level is fairly settled, that is, at the top of a rise which forms a plateau before the fall sets in, or at an unchanged high level.

Later similar results after ingestion of glucose have been reported by Klein [1925]. Gilbert, Schneider and Bock [1926] found similar results, but the blood was withdrawn only at intervals of 10-20 minutes.

Hansen thinks that these oscillations “represent the manner in which the blood-sugar regulation takes place.” Various observations in other work of mine have necessitated a further investigation into the occurrence of these oscillations. In particular, it was important to ascertain whether they really occur as a fairly constant phenomenon, which would seem most likely if they were a part of the intermediate sugar metabolism, or whether their appearance to some extent might be due to external factors resulting more or less from the experimental conditions under which the patients were examined. To get

some light on this question the experimental conditions were standardised as far as practicable and the technique was made as sensitive as possible.

In addition to the investigation on the oscillations of the blood-sugar values, this report also gives the result of experiments which were made in order to find out whether uniform blood-sugar curves might be obtained on different days after administration of the same amount of glucose. Bang [1914] has shown that in rabbits the administration of glucose causes a higher and more enduring rise of the blood-sugar in animals which have been starved for several days than it does in animals kept on ordinary diet. Later Traugott, Staub, Sakagushi [see v. Noorden and Isaac, 1927], Malmros [1928], Stenström [1928], Hagedorn [1921], Hansen [1923], Holst [1924], and others have shown that hyperglycaemia from the same doses of glucose or other carbohydrate does not always give the same curve, and that the course of the curve depends on the diet on the preceding days. The latter point is particularly emphasised by Stenström, who shows that the hyperglycaemia after glucose administration (the blood-sugar was determined only every  $\frac{1}{2}$  hour) is higher and more persistent after a period of fat and vegetable diet than it is after a period of normal diet.

#### TECHNIQUE.

The blood-sugar is determined by the Hagedorn-Jensen method. In order to make the analytical errors as small as possible the determinations were made in a particular way, which does not, however, differ from the original method in any essential point. With this exact method, the bleeding time and the pipette constitute the chief source of error. All samples of blood are taken in about the same length of time and in such a way that the blood enters the pipette freely, and the pipette is filled a little above the mark within 10–12 seconds and exactly adjusted within a few seconds more. The contents of the pipette are emptied carefully into the colloidal zinc solution in the usual way, after which the pipette is at once carefully cleaned.

The width of the lumen of the pipette may also be of some importance in measuring the content exactly, and I have therefore tried both tubular and oval pipettes of varying length. The same pipette has been used in most of the determinations here reported, namely, a capillary pipette measuring 22 cm. from point to mark. Otherwise the method has been carried out exactly as indicated by the authors. In most instances the precipitation of protein is done in direct connection with the withdrawal of blood. The sodium thiosulphate titration is done with a 2 cc. burette divided into 1/100 cc., and all titrations are made in 50–60 seconds and every analysis is completed within a few hours.

The patients had been lying perfectly quietly in bed for 10–15 minutes before the first sample of blood was taken (usually after 12–14 hours' fasting), and throughout the entire period of blood-taking, so that the curves have been in no way influenced by muscular work [Hagedorn, 1921; Hromadko, 1928; Weiland [see v. Noorden and Isaac, 1927], Hatlehol, 1924].

The experimental error of the method was determined on freshly drawn blood without anticoagulants and immediately after the withdrawal. A large number of tests, too voluminous to be reported here, showed a mean error of  $\pm 0.002$ .

For glucose administration 1 g. of pure anhydrous glucose per kg. of body-weight was used in 10 % aqueous solution.

Urinary sugar was determined qualitatively by Fehling's and Almén's tests, and quantitatively in some experiments by fermentation and in others by the Benedict-Osterberg [1918, 1921] method for total sugar determination.

#### EXPERIMENTS.

The experiments fall into the following groups:

- (1) blood-sugar in fasting non-diabetic patients;
- (2) blood-sugar in fasting diabetics;
- (3) blood-sugar after ingestion of glucose in non-diabetic patients;
- (4) blood-sugar during food absorption in diabetics;
- (5) blood-sugar curves on different days in non-diabetics on uniform diet.

##### (1) *Blood-sugar in fasting non-diabetic patients.*

This group comprises five patients with perfectly normal carbohydrate metabolism. The first blood was taken after 13 hours' fasting.

Fig. 1 *a* is typical of the blood-sugar findings in these five patients. In every instance the curve was quite smooth, without any "oscillation," as the variations in blood-sugar values do not exceed the limits of experimental error.

##### (2) *Blood-sugar in fasting diabetics.*

This group comprises 11 cases which fall into the following subdivisions:

- (a) six diabetics, not treated with insulin;
- (b) one diabetic with kidney lesion, not treated with insulin;
- (c) four insulin-treated diabetics.

The first blood was taken after 13 hours' fasting.

(a) *Diet-treated diabetics.* Fig. 1 *b* is typical of the blood-sugar findings in these six cases. In every instance it was found that, at perfect rest, the high fasting blood-sugar value decreases gradually and smoothly, without any "oscillations" in the curve during the 2 hours' observation.

(b) *Non-insulin-treated diabetes combined with nephritis.* There was practically no fall in the increased blood-sugar during 70 minutes of observation (initial value 0.285 %, final value 0.281 %), and there was no "oscillation" in the curve.

(c) *Insulin-treated diabetics.* Here are found two different forms of fasting blood-sugar curve: one, the most frequent (Fig. 1 *c*), in which the blood-sugar rises during fasting; and the other, more rare (Fig. 1 *d*), in which the blood-sugar falls during fasting. (The possible effect of a late insulin injection is excluded in this instance.) No "oscillation" was noticed in any of the cases examined.

In fasting diabetics, examination of the blood-sugar that has increased during the night [Hatléhol, 1924]—due to “physiological” conditions in diabetics during sleep—bears out the difference observed between insulin-treated diabetics and diabetics treated by dietary measures only, namely, that the blood-sugar in insulin-treated diabetics in most instances rises during 1–2 hours’ observation, whereas the blood-sugar in diet-treated diabetics falls in the same period.

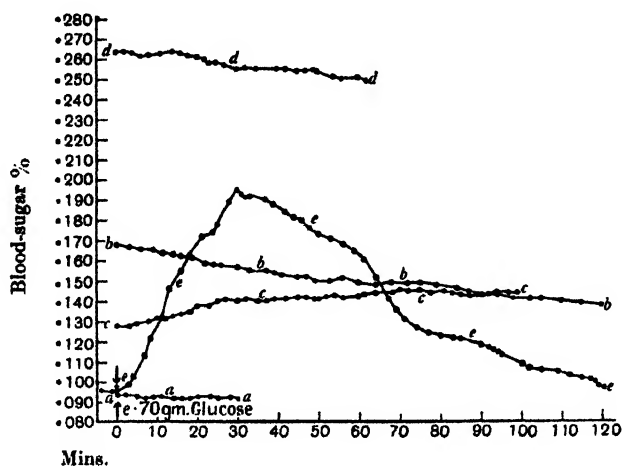


Fig. 1.

- a Blood-sugar in fasting non-diabetic.  
 b " " diet-treated diabetic.  
 c " " insulin-treated diabetic.  
 d " " " "  
 e " " after ingestion of glucose in non-diabetic.

### (3) *Blood-sugar after ingestion of glucose in non-diabetic patients.*

This group covers five patients with perfectly normal carbohydrate metabolism. The experimental conditions are exactly as already described. Fig. 1 *e* is typical of the blood-sugar curve in these patients. The rise itself, the fall and the waves of the individual curves cannot be discussed in detail in this paper, but no “oscillation” has been detected.

### (4) *Diabetics. Blood-sugar during food absorption in 24 hours.*

This group consists of two insulin-treated diabetics. One is examined at various times throughout the 24 hours; the other is examined only in connection with administration of insulin and intake of food. The blood-sugar findings in the first case are given in detail; the second patient shows a perfectly analogous course of blood-sugar curve. I was not able to find any “oscillation” of the blood-sugar curve in either of these two diabetics by determining the blood-sugar at various intervals throughout the 24 hours; there were only the well-known rises and falls and waves in connection with the insulin and the food intake.

J., male, 27 years. Patient has been treated with insulin for 12-14 months and is now getting 20 + 15 units of insulin "Leo." Patient was resting perfectly quietly in bed throughout the entire experimental period. The examination was made on 15. ix. 1927.

Time	Blood-sugar	Time	Blood-sugar	Time	Blood-sugar
hr. min.	%	hr. min.	%	hr. min.	%
Fasting		15.38	0-104	22.27	0-273
7.38	0-239	15.42	0-107	22.30	0-257
7.40	0-239	15.45	0-108	22.35	0-238
7.43	0-241	15.48	0-109	22.40	0-229
7.46	0-241	15.52	0-109	22.41	0-227
		15.56	0-111	22.42	0-226
20 units of insulin and 1 rusk		15.58	0-110	22.45	0-221
+ 1 cup of tea.		16.00	0-112	22.50	0-215
7.49	0-243	16.04	0-113	22.53	0-209
7.52	0-245	16.08	0-116	22.55	0-205
7.55	0-248	16.12	0-115	22.58	0-203
7.59	0-240	16.16	0-115	23.00	0-192
8.02	0-236	16.20	0-117	The patient slept during the following hours.	
8.06	0-228	16.24	0-118		
8.09	0-224	16.27	0-117	Next morning	
8.12	0-220	16.30	0-118	5.00	0-098
8.16	0-209	16.32	0-120	5.02	0-101
8.20	0-201	16.34	0-121	5.04	0-103
		16.36	0-121	5.08	0-109
Pause till 10.00, during which		16.40	0-122	5.09	0-111
the patient gets breakfast.				5.10	0-113
10.00	0-150	Pause for 3½ hours in which the		5.11	0-115
10.03	0-148	patient eats supper (at 18.30).		5.12	0-115
10.06	0-143	20.00	0-237	5.16	0-119
10.10	0-139	20.02	0-237	5.18	0-122
10.14	0-134	20.03	0-239	5.20	0-126
10.18	0-129	20.04	0-239	5.21	0-128
10.22	0-125	20.06	0-240	5.22	0-131
10.26	0-121	20.08	0-241	5.24	0-134
10.30	0-115	20.12	0-245	5.28	0-140
10.34	0-113	20.16	0-249	5.30	0-144
10.38	0-110	20.20	0-258	5.32	0-148
10.42	0-108	20.24	0-262	5.34	0-154
10.46	0-104	20.29	0-264	5.36	0-168
10.50	0-094	20.32	0-260	5.38	0-176
10.55	0-089	20.36	0-258	5.40	0-184
11.00	0-086	20.38	0-257	5.44	0-187
11.04	0-084	20.40	0-256	5.45	0-187
11.08	0-082	20.44	0-252	5.46	0-180
11.16	0-080	20.48	0-250	5.48	0-191
11.17	0-078	20.52	0-247	5.52	0-190
11.18	0-077	20.56	0-244	5.56	0-196
11.19	0-077	20.58	0-242	6.00	0-199
11.20	0-076	21.00	0-243	6.04	0-203
11.24	0-073	21.04	0-244	6.06	0-206
11.28	0-067	21.07	0-242	6.10	0-212
11.30	0-064	21.08	0-244	6.12	0-215
11.32	0-062	21.11	0-246	6.14	0-219
11.36	0-058	21.14	0-252	6.18	0-223
11.40	0-057	21.18	0-263	6.20	0-222
11.44	0-053	21.20	0-266	6.24	0-226
11.48	0-048	21.24	0-270	6.28	0-231
11.52	0-044	21.27	0-273	6.32	0-234
11.53	0-044	21.31	0-275	6.36	0-233
11.56	0-042	21.36	0-277	6.41	0-238
11.59	0-040	21.38	0-278	6.44	0-241
12.00	0-041	21.40	0-276	6.48	0-246
		21.44	0-279	6.52	0-248
Pause for 3 hours, during which		21.48	0-281	6.56	0-252
the patient gets dinner (at		21.53	0-283	7.00	0-254
13.00).		21.57	0-285	7.03	0-252
15.00	0-094	22.00	0-288	7.06	0-256
15.04	0-096	22.02	0-289	7.10	0-258
15.08	0-097	15 units of insulin "Leo."		7.14	0-256
15.12	0-099			7.18	0-256
15.16	0-097	22.04	0-291	7.22	0-258
15.20	0-099	22.10	0-293	7.26	0-260
15.22	0-098	22.14	0-289	7.28	0-261
15.24	0-099	22.18	0-283	7.29	0-262
15.28	0-101	22.23	0-278	7.30	0-262
15.32	0-102	22.24	0-276		

(5) *Blood-sugar curves on various days in non-diabetics on uniform diet.*

This last group gives the results of some experiments made in consequence of an investigation into the factors which regulate the carbohydrate metabolism. The object of the experiments was to find out whether it were possible with definite experimental conditions and uniform ingestion of glucose to obtain uniform blood-sugar curves on different days—uniform with regard to the rise of the curve as well as to its fall. It would take too much space to report the preliminary experiments which consisted in the daily intake of a definite amount of glucose irrespective of dietary balance, etc.; but the experiments showed that it was impossible in that way to obtain even approximately uniform curves. At once it was evident that the only possible way of obtaining uniform blood-sugar curves would be first to bring the organism into a stable equilibrium and then to proceed with the administration of glucose. I therefore tried whether I could obtain uniform curves in non-diabetic patients on ordinary mixed diet (without particular regard to its content of carbohydrates, proteins, fats, salts, etc.) by giving the same amount of glucose every fourth day; thinking that in the interval the individuals might return to the same equilibrium, but the following experiments proved this to be impossible.

In every experiment of this group the patients got a 10 % watery solution of 1 g. of glucose per kg. of body-weight after fasting 12 hours, and they had been resting perfectly quietly in bed throughout the experiment.

This group comprises altogether eight patients—with three to four uniform administrations of glucose in each case; but, as the findings are similar in all the cases, only two of them will be reported in detail.

In comparing the curves for each individual patient we find that, although they are on the whole fairly uniform with regard to approximately the same numerical rise, the curves differ rather markedly in the time of rise and in the waves on different days. As to the elimination of sugar by way of the urine, however, we find about the same total amount of glucose eliminated on each experimental day (the total amount of glucose determined by Benedict-Osterberg's method).

From these experiments it was evident that the pre-requisite for obtaining perfectly uniform curves on different days is that the patient is first balanced at an absolute equilibrium with regard to diet and all other factors and is then kept on exactly the same food every day as long as the experiment lasts. I have not yet had an opportunity of carrying out such experiments, but Fig. 2 supports this view. Throughout the entire experimental period this patient daily received practically the same amounts of carbohydrate, fat, protein, liquid and salts (his carbohydrate metabolism was not quite normal). Fig. 2 illustrates the blood-sugar findings in this case.

The three curves for this patient are very similar.

E. N., fem., 25 years. Neurasthenia.  
Ewald: 105 cc., 55/75. No objective  
signs apart from constipation

Blood-sugar

N. L., male, 39 years. Cardialgia and  
constipation. Ewald: 100 cc. 30/60.  
B.R. 125/90. Sahli 95 %

Blood-sugar

Date	... 16. ii.	19. ii.	24. ii.	27. i.	31. i.	4. ii.
Min.						
-5	0-078	0-080	0-080	0-086	0-085	0-081
-2	0-078	0-080	0-079	0-085	0-084	0-082
0	0-078	0-081	0-079	0-086	0-084	0-082
	Ingestion of 60 g. glucose in 600 cc. water			Ingestion of 60 g. glucose in 600 cc. water		
2	0-078	0-082	0-082	—	0-086	0-084
4	0-083	0-080	—	0-090	0-088	0-088
5	—	—	0-097	—	—	—
6	0-096	0-087	0-100	0-101	0-087	0-098
8	0-117	0-092	0-111	0-111	0-089	0-107
10	0-120	0-108	—	—	0-094	0-114
12	0-136	0-120	0-121	0-120	0-102	0-126
14	0-147	0-131	0-135	—	0-115	0-130
16	0-151	0-142	0-146	0-134	0-122	0-135
18	0-154	0-149	0-157	0-137	0-133	0-139
20	0-156	0-154	0-168	0-139	0-138	0-153
22	0-155	0-154	0-175	0-143	0-144	0-159
24	0-158	0-154	0-176	0-155	—	0-160
26	0-158	0-156	0-180	0-159	0-158	0-162
28	0-154	0-153	0-186	0-161	0-169	0-170
30	0-155	0-151	0-164	0-170	0-172	0-169
32	0-148	0-146	0-160	0-173	0-178	0-170
34	0-146	0-144	0-159	—	0-180	0-172
36	0-140	0-135	0-146	0-180	0-181	0-174
38	0-138	0-129	0-141	0-187	0-179	0-179
40	0-135	—	0-139	0-190	0-181	0-180
42	—	0-127	—	0-193	0-183	0-178
43	0-128	—	0-132	0-192	—	—
44	0-126	0-126	0-130	—	0-185	0-175
46	0-128	0-126	0-123	0-193	0-187	0-174
48	0-133	0-120	0-121	0-190	—	0-170
50	—	0-117	—	0-187	0-184	0-171
52	0-138	0-115	0-121	0-186	0-181	0-169
54	—	0-113	0-118	0-176	0-176	0-165
56	0-140	0-111	0-115	0-173	0-174	0-155
58	0-142	0-113	0-113	0-171	0-170	0-151
60	0-138	0-115	0-109	0-165	0-166	0-149
63	—	0-117	0-111	0-160	0-162	0-147
65	0-135	0-115	0-113	0-155	0-159	0-145
67	0-130	0-110	0-109	—	—	—
69	—	—	0-102	0-148	0-154	0-143
72	0-131	0-101	—	0-135	0-150	0-138
75	—	—	0-098	0-129	0-146	0-128
76	0-126	0-101	0-096	—	—	—
78	0-124	0-100	—	—	0-144	0-124
80	0-123	0-098	0-094	0-117	—	—
82	—	—	0-092	—	0-137	0-115
84	0-121	0-094	—	0-110	0-135	0-103
87	0-119	0-092	0-092	—	0-131	0-099
90	0-117	0-094	0-090	0-102	0-118	0-098
93	0-116	0-092	0-094	0-096	0-110	0-092
94	—	—	—	0-097	0-107	—
96	0-114	0-092	—	—	—	0-084
99	0-110	0-091	0-092	0-094	0-102	0-078
102	0-107	0-092	0-085	0-094	0-098	0-072
105	0-098	0-091	0-081	—	0-095	0-070
108	0-095	0-089	0-080	0-091	0-091	0-066
111	0-091	0-088	—	—	0-087	0-063
113	0-089	0-084	0-080	0-083	—	0-062
115	—	—	—	0-078	0-082	—
117	0-087	0-080	0-078	0-077	0-078	0-059
120	0-084	0-078	0-076	0-071	0-073	0-057

*Urine tests during these experiments.*

Date ...	16. ii.	19. ii.	24. ii.	27. i.	31. i.	4. ii.
Time (min.)	50 120	50 120	50 120	50 120	50 120	50 120
Diuresis (cc.)	320 475	400 400	580 280	230 265	300 350	330 300
Albumin	—	—	—	—	—	—
Glucose (mg.)	213 60	194 63	230 30	195 65	208 72	205 70
Total glucose eliminated (mg.)	273	257	260	260	280	275

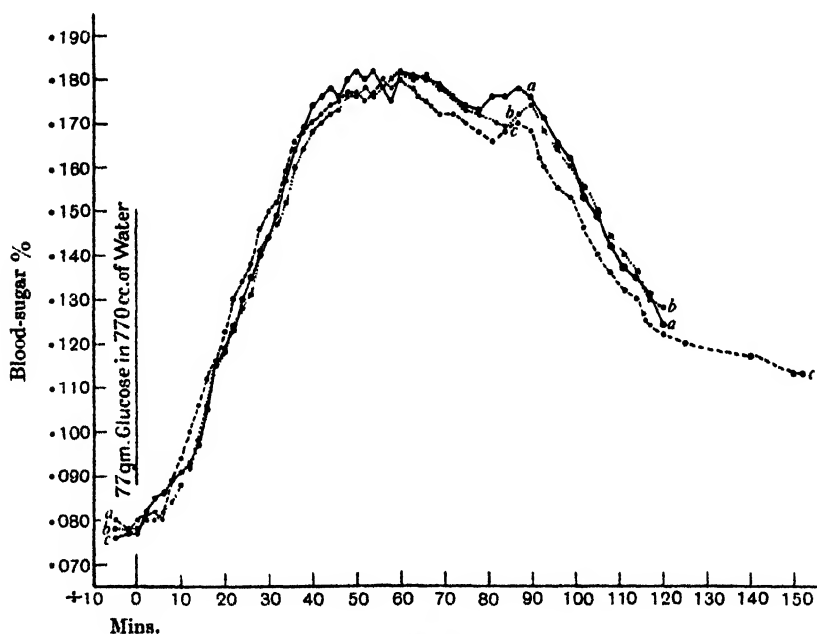


Fig. 2.

a 14. ii. 1927.

b 18. ii. 1927.

c 22. ii. 1927.

## SUMMARY.

1. In a number of determinations of the blood-sugar in fasting individuals at complete rest—made at intervals of 1–5 minutes for a period of  $\frac{1}{2}$ –2 hours—I have failed to find the previously described oscillations of the blood-sugar curve, either in normal individuals, in whom I found an almost horizontal curve, in insulin- and diet-treated diabetics, in whom I generally found a rising curve, or in diabetics merely treated with diet, in whom I found a falling curve, and in a diet-treated diabetic with advanced kidney insufficiency, in whom I found an almost horizontal blood-sugar curve.

2. In no instance did I find any “oscillations” in non-diabetics after ingestion of glucose or in diabetics at different times of the day after ingestion of food or administration of insulin.

3. I did not succeed in obtaining uniform blood-sugar curves on different days after ingestion of the same amount of glucose, although the experiments



were carried on for some time during which the individuals had plenty of fairly uniform mixed food. On the other hand, the amount of glucose eliminated in the urine in 2 hours was found to be about the same each day.

4. In a single instance I succeeded in obtaining uniform blood-sugar curves after ingestion of glucose on three different days. This was in a patient who on each day had about the same amounts of food, salts, liquid, etc.

#### REFERENCES.

- Bang (1914). Der Blutzucker.  
Benedict and Osterberg (1918). *J. Biol. Chem.* **34**, 195.  
——— (1921). *J. Biol. Chem.* **47**, 5.  
Gilbert, Schneider and Bock (1926). *J. Biol. Chem.* **67**, 629.  
Hagedorn (1921). Blodsukkerregulationen hos Mennesket.  
Hansen (1923). Investigation on the blood-sugar in man.  
Hatlehol (1924). *Acta Med. Scand. Suppl.* viii.  
Holst (1924). Undersøgelser over lette glykosurier.  
Hromadko (1928). *Zentrinn. Med.* **48**, 130.  
Klein (1925). *Shornik Lékařský*, **26**, 159.  
Macleod (1927). Kohlehydratstoffwechsel und Insulin.  
Malmros (1928). *Zentr. inn. Med.* **48**, 247.  
v. Noorden and Isaac (1927). Die Zuckerkrankheit und ihre Behandlung.  
Sakagushi, cit. v. Noorden and Isaac (1927).  
Staub, cit. v. Noorden and Isaac (1927).  
Stenström (1928). *Acta. Med. Scand.* **67**, 385.  
Traugott, cit. v. Noorden and Isaac (1927).  
Weiland, cit. v. Noorden and Isaac (1927).

# CLXXXV. THE ULTRA-VIOLET ABSORPTION SPECTRA OF URIC ACID AND OF THE ULTRA-FILTRATE OF SERUM.

By FRANK CAMPBELL SMITH.

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*(Received September 24th, 1928.)*

SINCE the ultra-violet absorption bands of uric acid are extremely sharp and well defined in as low a concentration as 1 mg. per 100 cc., it was considered necessary to measure accurately its absorption before that of the ultra-filtrate of serum.

The absorption spectrum of uric acid dissolved in water has been investigated by Dhéré [1909], who found two distinct bands at wave-lengths of Å 2920 and Å 2350. He was unable to measure the extinction coefficient, since at that time no suitable apparatus was available. He does not appear to have observed the change in the spectrum when the acid is dissolved in an alkaline solution.

Reinhard [1927] measured the absorption spectrum of the acid in water, but failed to observe the second absorption band. The present writer has measured the absorption spectrum of uric acid dissolved in water and in an alkaline solution, and has noted a marked difference in its absorption in each case.

## METHOD.

A Hilger quarter-plate spectrophotometer and rotating sector were used to measure the absorption. A condensed spark between tungsten steel electrodes was used as the source of radiation.

## EXPERIMENTAL.

The concentration of uric acid employed was 0.0026 %; this was chosen as a convenient strength to ensure that the extinction coefficient lay within the limits provided for by the rotating sector. The uric acid was dissolved in (1) distilled water, (2) a phosphate buffer solution at a  $p_H$  of 7.4, (3) a 0.1 % solution of sodium carbonate, (4) a 0.1 % solution of lithium carbonate. These solvents were employed in the comparison tube of the rotating sector.

The curves thus obtained are shown in Fig. 1. Their wave-lengths and extinction coefficients at the head and foot of the curve are given in Table I.

Table I.

		Wave-length (Å)		Extinction coefficient	
		Head	Foot	Head	Foot
Band $\alpha$	In water and at $p_H$ 7.4	2920	2600	1.46	0.28
	In carbonate	2950	2630	1.54	0.35
Band $\beta$	In water and at $p_H$ 7.4	2350	2210	1.2	0.66
	In carbonate	—	—	—	—

When dissolved in distilled water or in the buffer solution, the acid shows two distinct bands; when, however, it is dissolved in carbonate the second band disappears. At the same time, band  $\alpha$  moves towards the red, and the value of the extinction coefficient at the head and foot of the curve is slightly increased (v. Table I). The disappearance of the band is of interest in two connections, namely, (1) the possible variation in the structural formula of uric acid when dissolved in water and in carbonate; (2) the absorption spectrum of serum ultra-filtrate; this will be discussed below.

With regard to the first point, the difference in the absorption spectrum probably indicates a tautomeric change of the molecule.

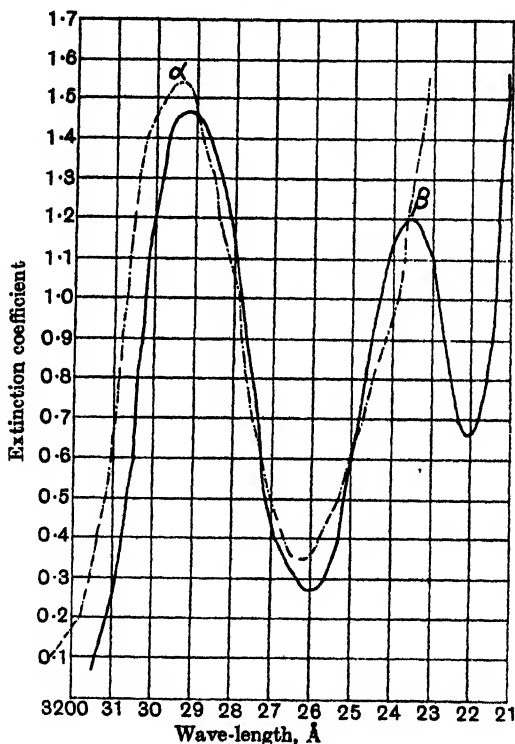


Fig. 1.

Interrupted line—uric acid dissolved in carbonate.

Continuous line—uric acid dissolved in water or phosphate buffer.

#### *Serum ultra-filtrates.*

To the best of the writer's knowledge, the ultra-violet absorption spectra of serum ultra-filtrates have not been investigated. The method of filtration has been described by him elsewhere [1928]. Three essential conditions must be observed, namely, (1) the filtration must be rapid, as it is difficult to keep serum filtrate sterile; the ultra-filtration of 10 cc. undiluted serum should not take more than 2 to 3 hours; (2) as large an area of membrane as possible

should be used; (3) the serum should be kept moving on the surface of the membrane. The pressure used need not be greater than 60 mm. Hg.

From 10 cc. of serum, 4-5 cc. of protein-free filtrate should be obtainable. The filtrate was not used unless the sulphosalicylic acid test was negative.

The results obtained in 14 cases (13 normal and 1 pathological) are tabulated below.

Table II.

Case no.	Wave-length (Å)		Extinction coefficient		Concentration of uric acid estimated from extinction coefficient (mg. per 100 cc.)	Remarks
	Head	Foot	Head	Foot		
1	$\alpha$ 2920	2700	2.2	1.32	3.9	
	$\beta$ 2350	—	3.4	—	—	
2	$\alpha$ 2920	2650	3.0	0.9	5.3	Fasting 14 hours
	$\beta$ 2300	—	3.0	—	—	
3	$\alpha$ 2920	2650	2.3	1.08	4.1	2-3 hours after breakfast
	$\beta$ 2300	—	3.0	—	—	
4	2920	2600	1.5	0.68	2.5	
5	2920	2620	2.4	0.92	4.3	
6	2910	2610	1.56	0.76	2.5	
7	2900	2700	2.4	1.32	4.3	2 hours after lunch. Well marked lipaemia
8	2910	2620	2.24	1.16	4.0	
9	2900	2620	1.54	0.92	2.7	
10	2900	2680	1.06	0.64	1.9	Fasting 13 hours
11	2920	2690	1.3	1.08	2.3	4 hours after large protein meal
12	2920	2770	1.08	0.96	1.9	4 hours after breakfast
13	2920	2600	2.4	0.8	4.3	
14	2950	2750	1.24	1.5	2.2	Advanced syphilis and carcinoma of liver

As an example of the results obtained, the curves of cases 3 and 14 are plotted in Fig. 2.

It is reasonable to suppose that the selective absorption shown is due to uric acid. As will be seen from the table, the time elapsing between the taking of a meal and the withdrawal of blood appears to have no effect.

If the concentration be estimated from the extinction coefficient at the head of the band  $\alpha$  (see Fig. 2), the average result agrees very fairly with the figures obtained by chemical analysis of normal blood. At this wave-length it may be assumed that little interference is caused by substances other than uric acid. As the wave-length becomes shorter, the absorption due to other substances becomes greater. As a result, the second band is of varying amplitude; for example, in cases 1, 2 and 3, of Table II it is definite, whereas in other cases, although always visible, it appears only as an inflexion at an approximate wave-length of Å 2350.

The absorption spectrum of uric acid present in the ultra-filtrate of blood-serum is similar to that of uric acid dissolved in water or in the buffer solution, and not to that of the acid dissolved in carbonate.

Work is now in progress to ascertain whether pathological conditions have any marked effect on the normal curve. No. 14 in Table II was obtained from a case presenting advanced syphilis and carcinoma of the liver. Band  $\alpha$  is seen as an inflexion and band  $\beta$  has disappeared (Fig. 2). This shows a wide deviation from the normal, which is evidently due to some abnormal substances present in the blood. The method of spectrophotometry may prove of value in the detection of such substances; further work in this connection is in progress.

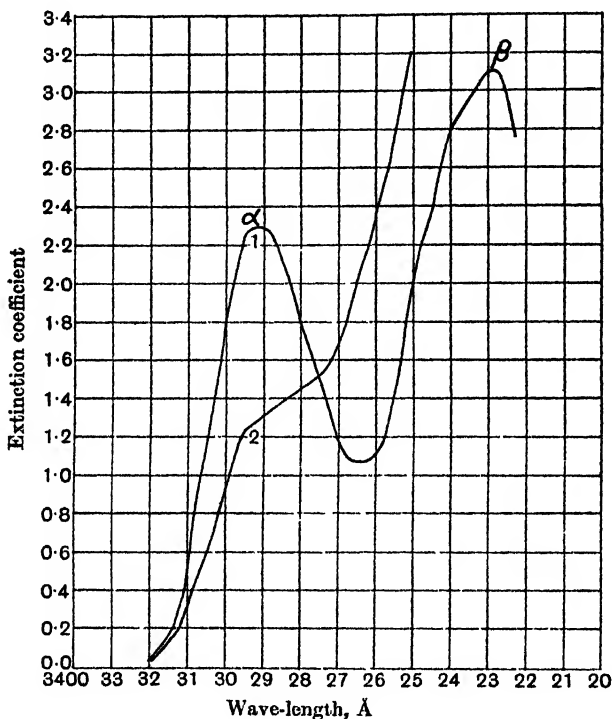


Fig. 2.

1. Normal curve.
2. Curve from pathological filtrate.

#### SUMMARY.

1. The ultra-violet absorption spectra of uric acid and of the ultra-filtrate of normal serum have been measured.
2. Uric acid, when dissolved in water, shows two sharp bands: when dissolved in carbonate, only one is visible. This possibly indicates a tautomeric change in the molecule.
3. It is reasonable to assume that the selective absorption shown by serum ultra-filtrates is due to uric acid.
4. The values given for the concentration of uric acid, as estimated by the amount of absorption, are in very fair agreement with those obtained by chemical methods.

5. A filtrate obtained from a pathological case has been investigated; it shows marked general absorption, thus differing widely from the filtrates of normal sera.

The writer wishes to express his indebtedness to Prof. H. E. Roaf, Dr J. R. Marrack and Dr L. F. Hewitt for their advice and assistance; further, to the Yarrow Research Fund of the London Hospital for defraying the expenses of this research.

#### REFERENCES.

- Dhéré (1909). *Recherches spectrographiques* (Fribourg).  
Reinhard (1927). *J. Gen. Physiol.* 11, 1.  
Smith (1928). *J. Physiol.* 65, *Proc. Physiol. Soc.*

## CLXXXVI. ALCOHOLIC FERMENTATION BY *ASPERGILLUS FLAVUS*, BREFELD.

By JOHN LEWIS YUILL.

(Received September 5th, 1928.)

*A. flavus* and *A. oryzae* present a number of at least superficial resemblances, and occur in nature under similar conditions. Thom and Church [1926] state that though the typical *A. flavus* may be distinguished from the typical *A. oryzae* with great contrast, yet, in a whole series of the naturally occurring organisms, the separateness of these two groups breaks down.

The latter mould has been shown by Yabuta [1912, 1916, 1924] to produce kojic acid from carbohydrates. The accidental discovery that a yellow-green *Aspergillus* belonging to the *A. oryzae* group produced crystals of calcium kojate when grown on cane-sugar solution in presence of chalk, led me to try if *A. flavus* also gave this compound.

I found no kojic acid produced by a typical strain of *A. flavus*, but the experiment led to the unlooked-for result of considerable alcohol production by this mould. Under the same conditions *A. oryzae* produced no alcohol.

A perusal of the literature fails to reveal any undoubted capacity among the *Aspergilli* of fermenting sugar to alcohol, when allowed to grow on a liquid with exposure to air, such as obtains in an ordinary flask culture. Small quantities are formed when the mycelium of certain strains is deliberately submerged in the sugary liquid, or when the air supply is otherwise deliberately withheld.

Pasteur [1876] experimented on the alcoholic capacities of *Penicillium* and *Aspergillus glaucus* (he later had some doubts as to the identity of this "*A. glaucus*"), and reached the conclusion that these moulds when deprived of free oxygen, as by submerging the germinating spores or the mycelium, would decompose sugar after the manner of yeast; but in full growth, with plenty of air, *Aspergillus* would not yield alcohol.

Wehmer [1910] gives a good account of the question of alcohol production by *Aspergillus*, and concludes that the possibility of some strains producing alcohol in more than mere traces cannot be altogether brushed aside, but the matter needs further investigation.

Waksman [1922] gives a bibliographic review up to 1922 of the enzymes of micro-organisms. The conclusion that the anaerobic respiration of *A. niger*, when the mycelium is submerged, takes the nature of alcoholic fermentation, is supported by Kostytschew's experiment in which a three day-old mycelium,

submerged by glass beads and deprived of further air, produced alcohol and carbon dioxide in practically the usual yeast ratio. Zymase is not specifically attributed to other *Aspergilli* in this review.

Thom and Church [1926], in a summary of the enzymic and fermentative activities of the *Aspergilli*, recall that, in the case of the saké fermentation, the earliest workers attributed the alcohol production to *A. oryzae*, but that yeasts were subsequently proved to produce the alcohol.

Oppenheimer [1926] finds that *Aspergillus* can be induced to give anaerobic alcoholic fermentation, with certain precautions, but that aerobically absolutely no alcoholic fermentation results. He upholds Wehmer in questioning Elfing's statement of the production of 4.2 % alcohol by "*P. glaucum*." Oppenheimer states too that anaerobically the mould fungi are extremely sensitive to acids, and that one way of overcoming this is by keeping the solution neutral with chalk.

*A. flavus* does not appear to have received special consideration in this connection.

The very definite production of ethyl alcohol by *A. flavus* in the experiments about to be described, under conditions which would ordinarily be termed aerobic, is not necessarily at variance with the findings in the literature cited above. The conditions were exactly the same as those which suffice for such typically aerobic changes as the oxidation of glucose to gluconic acid and citric acid by *A. niger*. Indeed *A. flavus* itself produces some citric acid in these conditions. Whatever differences there are in the access of the mycelial cells of the two fungi to the air are intrinsic to the respective habits of growth of the two species. As noticed below, the mycelial felt of *A. flavus*, under these conditions, is rather remarkable for its apparent thickness and open texture; and since its upper surface remains practically only just above water throughout its growth, a certain degree of anaerobiosis must be enjoyed by the cells of its lower layers. Thus, if it is right to connect the alcohol production with this feature of the mould's growth, we have merely another instance of the essentially anaerobic nature of the alcoholic fermentation.

#### EXPERIMENTAL.

The culture used throughout was the type culture of *A. flavus* from the National Collection of Type Cultures at the Lister Institute.

The medium selected was based on Currie's formula quoted below: a medium originally devised for optimum citric acid yields with *A. niger* [Currie, 1917].

Sucrose	125-150 g.	} per 1000 cc.
NH <sub>4</sub> NO <sub>3</sub>	2.0-2.5 g.	
KH <sub>2</sub> PO <sub>4</sub>	0.75-1.0 g.	
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.20-0.25 g.	
HCl to p <sub>H</sub> 3.4-3.5 (5-4 cc. N/5)		



Small conical flasks, filled to a depth of about half an inch with the above medium, were used, and a little chalk, autoclaved separately, was added to each. This procedure is necessary to avoid undesirable changes in the medium. Fernbach [1916] states that otherwise partial and even complete precipitation of phosphates may occur at the temperature of sterilisation.

After inoculation from mature subcultures of *A. flavus*, the flasks were incubated at 28° for 10–12 days.

The cultures early acquired a rather heavy, somewhat fruity alcoholic smell, and bubbles of gas were disengaged from the chalk. The growth of the mycelium was rapid and in most cases it remained free from spores. The mycelium, though not dense, was unusually thick, reaching 3 mm. in 10 days. Ordinary pressure from the fingers expressed a large volume of liquor from the mould; microscopical examination of a cross-section showed an open network of hyphae. This mode of growth is doubtless important in relation to alcohol production.

The liquor from the cultures, augmented with the liquor expressed from the moulds, was filtered and distilled. The distillate gave the iodoform and dichromate reactions. After redistillation, saturation with potassium carbonate, and separation, a considerable fraction distilled between 78° and 79° and gave the tests for ethyl alcohol.

The fermented liquor also contained a considerable quantity of calcium salts in solution. Part of this is accounted for by calcium citrate. This was precipitated on boiling, and the presence of the acid confirmed by Denigès's test. Liquors from cultures which spored contained more citrate than those which did not spore. The latter, however, invariably contained in solution much calcium in the form of compounds precipitable by strong alcohol. Further work on the calcium salts in solution is in progress.

A series of cultures was also studied to obtain some idea of the rate of production of alcohol by *A. flavus* and of the quantity of alcohol produced. The solution from the cultures was distilled and the distillate tested for alcohol by the colorimetric method of Bertrand and Thomas [1920]; the total reducing substances and residual cane-sugar were estimated by the decoloration method, before and after hydrolysis with HCl. All the cane-sugar appeared to be inverted by the eighth day.

The data are summarised in the following table.

Differences with regard to sporulation appear to account for the major irregularities in the results, whilst in any such series of cultures, where ideas of continuous changes can only be got from examination of different individuals, some irregularities are to be expected.

Table I.

Culture	Age in days	Wt. of wet mould (g.)	Wt. of dry mould (g.)	Sugar con- sumed (g.)	Alcohol pro- duced (g.)	g. alc. per 100 cc. liquor	g. alc. per 100 g. sugar consumed	Condition as regards sporu- lation
1	2½	8.0	0.20	1.55	0.11	0.21	7.1	No spores
2	3½	9.2	0.35	2.35	0.18	0.32	7.6	"
3	4½	9.6	0.38	3.35	0.62	1.19	18.5	"
4	5½	10.7	0.41	3.75	0.90	1.71	24.0	"
5	6½	10.7	0.72	5.31	1.00	1.96	18.8	"
6	6½	—	1.20	5.95	0.62	1.26	10.4	Sporing
7	7½	12.5	0.50	5.25	1.25	2.43	23.8	No spores
8	8½	9.9	1.50	6.73	0.38	0.81	5.6	Sporing
9	9½	14.0	1.00	6.85	1.00	2.02	14.6	No spores
10	9½	12.9	1.00	—	1.17	—	—	"
11	10½	10.3	1.80	6.85	0.25	0.53	3.6	Sporing
12	12½	15.7	0.87	7.49	1.30	2.82	17.3	No spores
13	13½	14.3	0.77	7.33	1.30	2.76	17.7	"
14	22½	—	0.97	7.80	0.50	1.11	6.40	"
15	22½	12.3	2.19	7.85	0.002	0.004	0.03	Sporing
16	23½	10.0	1.60	7.85	0.00	0.00	0.00	"

## SUMMARY.

*A. flavus* was not found to produce kojic acid under conditions in which *A. oryzae* is known to give it.

In presence of chalk, *A. flavus*, without any precautions to exclude air from the cultures, produces considerable quantities of ethyl alcohol.

In a series of cultures the alcohol amounted to 2–3 % in the fermented liquor, and represented 15–25 %, by weight, of the sugar consumed.

Under the same conditions, *A. oryzae* was not found to produce any alcohol.

During the fermentation with *A. flavus* considerable quantities of chalk are dissolved, some calcium citrate is formed and some soluble calcium salts precipitable by alcohol.

When the mould forms spores, the yield of alcohol is much reduced and the quantity of citrate increased.

## REFERENCES.

- Bertrand and Thomas (1920). Practical biological chemistry (Bell), 284.  
 Currie (1917). *J. Biol. Chem.* **31**, 15.  
 Fernbach (1916). *J. Inst. Brewing*, **22**, 358.  
 Oppenheimer (1926). *Die Fermente*, **2**, 1585, 1586.  
 Pasteur (1876). *Études sur la bière* (Paris), 102.  
 Thom and Church (1926). *The Aspergilli* (Williams and Wilkins).  
 Waksman (1922). *Abstr. Bact.* **6**, 298.  
 Wehmer (1910). *Lafar's Technical mycology* (Griffin), **2**, 367, 368.  
 Yabuta (1912). *J. Coll. Agric. Imp. Univ. Tokyo*, **5**, 51–58.  
 — (1916). *J. Chem. Soc. Tokyo*, **37**, 1185, 1234.  
 — (1924). *J. Chem. Soc.* **125**, 575.

# CLXXXVII. A NOTE ON THE LACTIC ACID AND GLYCOGEN CONTENT OF KIDNEY CORTEX.

By JAMES TUTIN IRVING (*Beit Memorial Research Fellow*).

*From the Biochemical Laboratory, Oxford.*

(Received October 1st, 1928.)

DURING the course of work already described [1928] some interesting observations were made on the metabolism of the rabbit's kidney which appeared to justify further investigation. The present experiments have been undertaken with the object of comparing the initial values of the glycogen and lactic acid content of the cortex with those obtained after the tissue had been incubated. The methods employed and the results obtained are described below.

## EXPERIMENTAL.

*Glycogen*<sup>1</sup>. The method of estimation used was a micro-modification of that of Pflüger. 2 g. of kidney tissue were heated in a small centrifuge tube at 100° with 2 cc. of 60 % potassium hydroxide for 2 hours. At the end of that time, the tube was cooled and 6.2 cc. of absolute alcohol and a pinch of NaCl were added; the precipitate, usually fairly bulky, was centrifuged down, and the supernatant fluid decanted and discarded. The precipitate was then usually teased up in a small amount of 70 % alcohol with a thin glass rod, the end of which was ultimately broken off into the suspension to avoid any loss. The precipitate was again centrifuged down, and the supernatant fluid poured away. 6 cc. of a 2.2 % hydrochloric acid solution were now added and the tube was heated in a boiling water-bath for 4 hours. The contents were then neutralised and filtered, and the filtrate was made up to 20 cc.; sugar estimations were carried out on 2 or 5 cc. by the method of Hagedorn and Jensen.

The following table shows the degree of accuracy of this method on pure glycogen and on glycogen added to kidney tissue.

Table I. *Estimation of pure glycogen.*

	mg.	mg.
Amount taken	0.95	1.77
Amount recovered	0.91	1.71

*Recovery of added glycogen from kidney cortex.*

	mg.	mg.	mg.
Glycogen content of 2 g. cortex	1.36	0.90	1.26
Glycogen content of cortex plus added glycogen	2.33	1.70	1.93
Glycogen added	0.95	0.82	0.75
Glycogen recovered	0.97	0.80	0.67

<sup>1</sup> The writer is indebted to Prof. C. Lovatt Evans, F.R.S., and to Dr W. K. Slater for much useful information on the properties and estimation of glycogen.

The use of one tube for the whole estimation avoided what had been found a frequent source of error, namely, adhesion of the precipitated glycogen to the walls of the flasks previously employed. The figures given below all express glycogen in terms of glucose.

*Lactic acid.* The lactic acid was extracted and estimated by a similar routine to that previously described [1928]. A weighed amount of cortex (usually about 2 g.) was ground with sand in a mortar containing 5–7 cc. of 6 % trichloroacetic acid as protein precipitant. The resulting suspension was made up to 10 or 15 cc. and centrifuged; lactic acid was extracted from 5 cc. of the supernatant fluid with ether and the estimation was carried out by the method of Friedmann, Cotonio and Shaffer [1927].

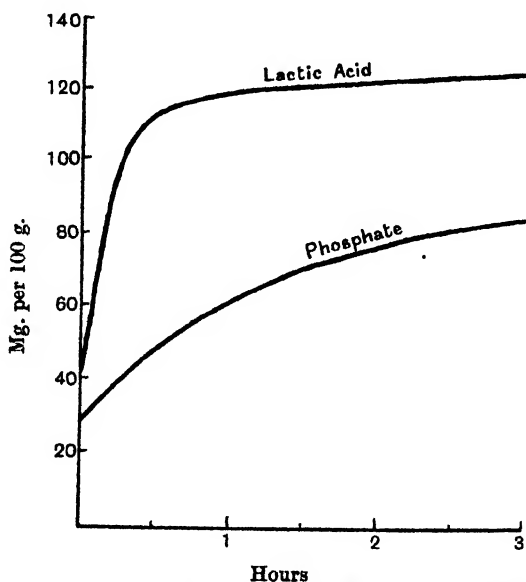


Fig. 1. The production of lactic acid and free phosphate in incubated kidney cortex.

*The initial values* were obtained as follows. The rabbit was killed by a blow on the back of the skull, and the left kidney, as being the most accessible, was dissected out as rapidly as possible and dropped into freezing mixture at  $-15^{\circ}$ . The time taken by this operation varied from 22 to 36 seconds. After the kidney had frozen solid, one-half of the cortex was removed and placed in a cooled weighed beaker containing 5–7 cc. of frozen trichloroacetic acid, and the rest of the cortex was dropped into a cooled weighed centrifuge tube containing 1.5 cc. of frozen 60 % potassium hydroxide. In both samples the cortex was mixed as thoroughly as possible with the frozen acid and potash. After the vessels had been again weighed, that containing the sample for glycogen estimation was plunged into boiling water and, after the tissue had disintegrated, more potassium hydroxide if necessary was added. The lactic acid sample, still frozen solid, was transferred from the beaker to a cooled

mortar and rapidly ground with sand. When the hash had completely melted it was transferred to a small cylinder and made up to the requisite volume, the procedure being then as described above.

*The final values* were obtained from the remaining kidney. This was removed from the animal immediately after the left kidney had been placed in the freezing mixture. Slices of the cortex were put in a small stoppered weighing bottle and incubated at 37.5° for 2 hours. The procedure was then as described above.

In order to show that the above technique was justified, final values were determined on the right and left kidneys of the same animal. The results in Table II show that for this purpose the two kidneys appear to be identical.

Table II. *Comparison of the final values of lactic acid and glycogen of the right and left kidneys.*

	Right	Left
Glycogen (mg. per 100 g.)	37	34
Lactic acid (mg. per 100 g.)	142	146

#### RESULTS.

*The production of lactic acid.* During the work previously described [1928], it was frequently noted that the production of lactic acid was a matter of some rapidity and it was soon found that the size of the initial value depended entirely on the speed with which the kidney was removed from the body. In this respect this organ closely resembles muscle; Exp. 7 in Table III and Fig. 1, the latter the result of an experiment undertaken with Dr H. D. Kay, clearly illustrate this fact. The initial values obtained are shown in Table III but it is very improbable that these represent the true resting lactic acid content: this value must in the normal kidney be but a mere trace [cf. Davenport and Davenport, 1927].

The final values obtained after incubation were usually fairly constant, varying between 130 and 160 mg. per 100 g., provided that comparable techniques as regards the handling of the animals were used. Thus Exp. 5 is of interest as showing the effect of ether anaesthesia and the resulting muscular activity on, presumably, the blood-lactic acid.

*Glycogen.* A perusal of the literature has shown that little is known concerning the glycogen content of the kidney. Its occurrence in this organ was first noted by Claude Bernard, and a few writers [*e.g.* Erlich, 1883; Pashutin, 1884] also identified it by histological methods. More recently Brown and Greene [1918] reported its presence but gave no figures. Fichera [1904], however, found none in normal tissue, and small amounts only after phloridzin or experimental glycosuria. Junkersdorf and his co-workers [1924, 1925] are, to the writer's knowledge, the only authors who quote quantitative results, but they worked solely with phloridzinised dogs. The values they reported varied between 39 and 180 mg. per 100 g.

The values obtained by the present writer are given in Table III. It will be seen that the initial figures show a lack of constancy and that, with one exception, the differences between the initial and final values are small, or else no difference exists. Since it is not unlikely that these small differences are due to flaws in the technique, it may be doubted whether the values obtained represent glycogen at all. The glycogen content of other tissues (*e.g.* muscle [Simpson and Macleod, 1927]) falls very rapidly under similar conditions; the behaviour of kidney-glycogen, however, appears to resemble more closely that of brain [Holmes and Holmes, 1926].

Table III. *Lactic acid and glycogen content of kidney cortex before and after incubation at 37.5° for 2 hours.*

Exp. no.	Lactic acid (mg. per 100 g.)		Glycogen (mg. per 100 g.)		Lactic acid increase (mg. per 100 g.)	Glycogen decrease (mg. per 100 g.)
	Initial	Final	Initial	Final		
1	68.5	161	103	57	92.5	46
2	60	129	102	84	69	18
3	82	170	40	44	88	0
4	45	132	50	40	87	10
5	145*	233	33	38	88	0
6	52	142	68	51	90	17
7	133†	144	—	—	11	—

\* Animal anaesthetised with ether. Kidney frozen *in situ* with ethyl chloride.

† Experiment to illustrate the effect of slowness in removing kidney.

Of greater interest is the evidence derived from a comparison of these figures with those obtained for lactic acid. The discrepancy which exists between the lactic acid produced and the glycogen changes observed suggests that (with the exception of Exp. 1) very little if any lactic acid comes from this source: the remaining acid presumably being derived from some non-glycogen precursor.

In seeking for other precursors of lactic acid, the first which suggested itself was free sugar. The easiest way to test this was to make use of a fact already described [Irving, 1927] that kidney tissue after freezing and subsequent thawing has completely lost its power of degrading glucose. Experiments were therefore carried out as follows: the left kidney was removed as quickly as possible and frozen, while the right kidney was removed at the same time but not cooled. The left kidney, when frozen solid, was cut in half; the cortex from one half was treated as usual for the initial value, while that from the other half was incubated for 2 hours. The cortex from the right kidney was simultaneously incubated as a control. The results which are shown in Table IV indicate that a small part of the lactic acid formed may have its origin in glucose, but the major part comes from some other precursor.

Table IV. *Lactic acid content (mg. per 100 g.) of normal and previously frozen kidney cortex before and after incubation at 37.5° for 2 hours.*

Exp. no.	Initial	Final	
		Frozen cortex	Normal cortex
1	51	115	145
2	49	108	—

The most probable of these might be supposed to be a lactacidogen-like substance; but, as stated by Kay [1928], experiment has shown that such an origin is extremely improbable, the rate of breakdown of the naturally occurring phosphoric esters appearing to be entirely independent of that of the production of lactic acid. This is shown in Fig. 1. It is of course possible that the large and unrelated changes seen in the free phosphate content of incubated kidney tissue may obscure possible changes in free phosphate connected with lactic acid production, but at present no decisive answer is available.

In conclusion, it is interesting to speculate on the significance of the rapid production of lactic acid which occurs on removal of the kidney from the body. The similar changes which occur in muscle under these conditions are the deranged chemical transformations which are normally associated with contraction. To what process in the normal kidney this chemical change is related is as yet completely unknown; but there can be no doubt that the rapid production of lactic acid in the excised kidney must represent the first onset of autolysis and may be but a caricature of any process in the normal organ. It is of interest to note, without in any way implying a teleological argument, that McCance [1924] has found that the optimum reaction for certain autolytic changes is in the neighbourhood of  $p_H$  6.0.

The production of lactic acid from preformed compounds in the kidney, on analogy with the changes in muscle, is probably an intermittent phenomenon; it is therefore likely that this transformation is associated with some definite specialised function, while the utilisation of glucose, a regular linear process, is related more to the continued metabolism and existence of the cells themselves.

#### SUMMARY.

The production of lactic acid in excised kidney cortex is, like that in muscle, a reaction of some rapidity. Since under these conditions the glycogen content changes but little, it is deduced that the major part of the lactic acid must be formed from some other source. It is likely that glucose may be one of the precursors, but the origin of the bulk of the acid is unidentified.

It is probable that the glycogen figures obtained are contributed, to a large extent, by non-glycogen substances.

The writer is indebted to Prof. R. A. Peters for his continued advice and interest in this work.

The expenses of this research were defrayed by a grant from the Government Grant Committee of the Royal Society.

## REFERENCES.

- Brown and Greene (1918). *Amer. J. Physiol.* **45**, 570.  
Erich (1883). *Z. klin. Med.* **6**, 33.  
Davenport and Davenport (1927). *Proc. Soc. Exp. Biol. Med.* **25**, 177.  
Fichera (1904). *Beitr. path. Anat. allgem. Path.* **36**, 273.  
Friedmann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.  
Holmes and Holmes (1926). *Biochem. J.* **20**, 1196.  
Irving (1927). *Biochem. J.* **21**, 880.  
—— (1928). *Biochem. J.* **22**, 964.  
Junkersdorf and Bickenbach (1925). *Arch. ges. Physiol.* **208**, 638.  
Junkersdorf and Kühn (1925). *Arch. ges. Physiol.* **208**, 617.  
Junkersdorf and Török (1924). *Arch. ges. Physiol.* **204**, 127.  
Kay (1928). *Biochem. J.* **22**, 855.  
McCance (1924). *Biochem. J.* **18**, 486.  
Paschutin (1884). *Centr. med. Wissensch.* **22**, 689.  
Simpson and Macleod (1927). *J. Physiol.* **64**, 255.



# CLXXXVIII. THE FLUORESCENCE OF SOME VITAMIN A-CONTAINING FATS.

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*(Received October 23rd, 1928.)*

WHEN a beam of light from a quartz mercury vapour lamp, completely enclosed so that the only light emitted has to pass through a filter cutting out nearly the whole of the visible portion of the spectrum, is allowed to fall on to any object in a darkened room, a more or less characteristic fluorescence is usually observed. Metals, such as gold, silver, iron, etc., are quite non-fluorescent and take on the appearance of black velvet when observed in this light, but many other substances emit quite a bright coloured light. Several references are to be found in the literature suggesting the application of this phenomenon in various branches of analysis (*e.g.* Gerngross and Schulz [1927]; Popp [1927]; Volmar [1927]), and perhaps the most striking observation is that butter can be distinguished from margarine by the quite dissimilar fluorescence. Butter shows a fairly bright yellow colour, but margarine in this light is definitely blue.

## I. APPARATUS FOR THE MEASUREMENT OF FLUORESCENCE.

Work on the use of fluorescence in analysis has been hindered by the difficulty of describing, without the help of a demonstration, the phenomena that have been observed. This difficulty can be overcome by giving some sort of measurement to the fluorescence, and in the present work we have used an adaptation of the colorimeter described by Guild [1927] and manufactured by Adam Hilger & Co., Ltd. This instrument is used for the standardisation of colours in terms of the three additive primaries: red, green and blue. For that purpose the coloured sample is illuminated by a standard lamp, and the light from it is viewed in an eyepiece and matched by the light from the primaries, which fills a rectangle with one side adjacent to the rectangle of light from the coloured sample. The direct reading obtained gives a measure of the colour in units (up to 100 each) of red, green and blue, but this reading is dependent on the sensitivity of the observer's eye to the different primaries, and fairly wide variations are obtained between the readings of different individuals. These can be eliminated by referring all the readings to the particular observer's reading on a standard white disc supplied with the instrument. The final expression obtained gives (i) the "shade," which is the measure of the total light reflected from the sample when the total light

reflected from the standard white disc illuminated similarly = 100, and (ii) the "quality," which is the analysis of the total light reflected into percentages of red, green and blue, assuming that the analysis of standard white is 33.3 % red, 33.3 % green and 33.3 % blue.

The colorimeter was housed in a dark room, and in making fluorescence readings the sample of fat was illuminated by filtered ultra-violet light instead of by white light from the standard lamp. The fluorescence was viewed in the eyepiece and matched by the additive primaries exactly as are the colours illuminated by white light, and expressions of "shade" and "quality" were obtained by referring the readings to the same observer's readings on "standard white" illuminated by the ordinary light. The "shade" figure is a measure of the brightness of the fluorescence, in purely arbitrary units. The "quality" figure has exactly the same significance as in ordinary colour readings.

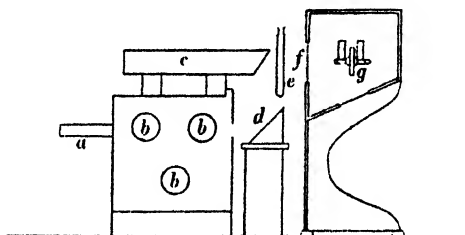


Fig. 1. *a*, eyepiece; *bbb*, knobs controlling colour filter apertures; *c*, stand carrying skip with fat sample; *d*, mirror reflecting sample; *e*, watch-shaped quartz flask filled with water; *f*, filter cutting out most of visible light from mercury vapour lamp; *g*, mercury vapour lamp.

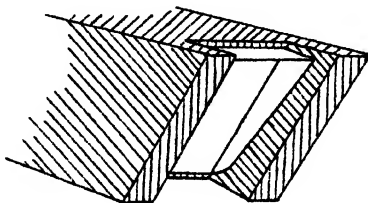


Fig. 2. Detail of stand and skip for fat sample.

Throughout the work recorded in this paper, all the readings were carried out by two observers, and unless good agreement was obtained in the analysis, the result was rejected.

In our adaptation of the colorimeter to the measurement of fluorescence, the apparatus was arranged as shown in the diagram, Fig. 1.

It is important that the sample whose fluorescence is to be examined should be illuminated by filtered ultra-violet light at fairly close range, otherwise the fluorescence as seen in the eyepiece is only feeble, and cannot be matched with accuracy. The housing of our ultra-violet lamp (the Hanovia analytical quartz

lamp) did not make this easy, but the difficulty was overcome by reflecting the fluorescent light into the eyepiece by means of an ordinary mirror. The sample of fat was pressed into a small metal skip  $1'' \times 3\frac{3}{4}''$ , and smoothed to give a plane surface. The skip was fixed into a slot on the end of a wooden stand which could always be placed in exactly the same position on top of the case of the colorimeter, in a line parallel with the axis of the eyepiece, and in the same vertical plane. The arrangement of the skip and the stand is seen in detail in the diagram, Fig. 2. The mirror also was held in a thick wooden stand (to protect it from the heat of the lamp), and a watch-shaped quartz flask (8 mm. thick), filled with water, was interposed between the lamp and the fat sample, so that the latter should not become unduly heated by the radiation from the lamp. The whole apparatus could be dismantled and reassembled exactly as in the original positions.

This arrangement is admittedly susceptible of considerable improvement, but it has enabled us to make several measurements of interest, and to express our results in an understandable form. The standard lamp and the light source to the primaries are fitted with variable resistances, and in our colour readings we have used 112 volts and 120 volts respectively across the two lamps. In the fluorescence readings we did not at first control the voltage across the mercury vapour lamp, but later it appeared to be necessary because of fluctuations in the current supply, and the voltage across the lamp and fixed resistance attached was set at 112.

## II. THE FLUORESCENCE OF THE UNSAPONIFIABLE MATTER FROM COD-LIVER OIL.

Peacock [1926] reported that cod-liver oil exhibits a brilliant "golden" fluorescence in filtered ultra-violet light, and that exposure to white light of sufficient intensity results in the gradual disappearance of the fluorescence. From the observations that the fluorescence persists some time after the oil has ceased to give the arsenic trichloride test for vitamin A, and that, after the fluorescence has been entirely destroyed, if the oil is stored for several months in the dark, partial recovery of fluorescence takes place, he concluded that the destruction of vitamin A and the destruction of fluorescence are not identical phenomena. We have carried out many further experiments, not reported in the present paper, and we find that though there is a very close association between vitamin A and a characteristic fluorescence, certain discrepancies, such as those observed by Peacock, do occur. We have not yet observed any fat containing vitamin A that does not also show the fluorescence associated with it, and it seems that an explanation of the discrepancies may be that, while vitamin A is actually a brightly fluorescent substance, in certain circumstances other substances may be formed in oils, giving a fluorescence somewhat similar to that due to the vitamin. There does not appear to be any connection between fluorescence and vitamin D.

When a little unsaponifiable matter from cod-liver oil is added to a solid fat, the brightness of the fluorescence is increased, and the colour of the fluorescence is made less bluish. In Table I and Figs. 3 and 4, the effect of the addition of unsaponifiable matter from cod-liver oil upon the fluorescence of two fats is shown. The two fats used were hardened coconut oil and jus. For margarine manufacture, jus (pure rendered beef fat) is separated into two fractions of different melting point range. The lower melting fraction is "oleo," the higher is "oleo stearine." Jus normally gives only a dull fluorescence. It is seen how with successive additions of unsaponifiable matter the brightness is increased and the percentage of blue in the analysis

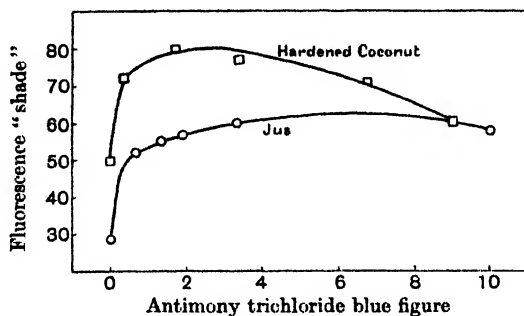


Fig. 3.

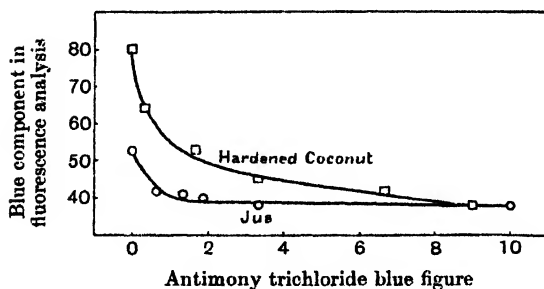


Fig. 4.

of the emitted light is diminished. As would be expected, the brightness reaches a limit after a certain amount of unsaponifiable matter has been added, and then begins to fall off slowly. If the fat were perfectly transparent to the exciting rays and the emitted light, it would be observed, when regarded from the side, that with successive additions of unsaponifiable matter the brightness would increase for a while through the whole depth of fat. Then a distinct zone of fluorescence would appear and the fat below this would not fluoresce, because the whole of the fluorescence-exciting rays had been absorbed in the depth of the zone. With further additions the zone would become more and more shallow until confined to a layer at the surface. Regarded from the surface, the limit of brightness would be reached as soon as the fluorescent

zone appeared, and after a while further additions of unsaponifiable matter would slightly depress the fluorescence, because of the opacity of the added material to the emitted light. The unsaponifiable matter from cod-liver oil is dark in colour, and, as will be seen later, the addition of a pigment depresses the brightness of the fluorescence of a fat.

With an imperfectly transparent fat, the fluorescence-exciting rays are absorbed, not only by the fluorescent substance, but by the fat, and the fat also absorbs part of the emitted light. The limit of brightness will be reached when a fairly large increase in the amount of unsaponifiable matter present will make so small a difference to the depth of the fluorescent zone that the opacity of the fat ceases to be an important factor.

Table I. *Effect of the addition of unsaponifiable matter upon the fluorescence of a fat.*

	Antimony trichloride "blue" figure*	Shade	Quality		
			Red	Green	Blue
Jus ... ..	—	29	14	33	53
Jus + unsap. matter from c.-l.o. ...	0.66	52	19	39	42
Ditto ... ..	1.33	55	20	39	41
Ditto ... ..	1.90	57	20	40	40
Ditto ... ..	3.33	60	21	41	38
Ditto ... ..	10.0	58	22	40	38
Hardened coconut oil ... ..	—	50	5	14	81
Hardened coconut oil + unsap. matter from c.-l.o. ... ..	0.33	72	11	25	64
Ditto ... ..	1.67	80	15	32	53
Ditto ... ..	3.33	77	17	38	45
Ditto ... ..	6.66	71	19	39	42
Ditto ... ..	9.0	60	20	42	38

\* The "antimony trichloride blue figures" are determined in a Lovibond tintometer according to the method described by Carr and Price [1926]. 0.2 cc. of a wt./vol. solution of the oil or fat in chloroform is run into an 8 mm. test-tube, and 2 cc. of a saturated solution of antimony trichloride in chloroform are added. The blue colour obtained is matched by the Lovibond glasses, and the result calculated to a 20 % solution of oil. The blue figure of 0.3 may be taken as that of good butter-fat (determined from readings on the unsaponifiable matter), and the average for a large number of cod-liver oils purchased in various parts of England this year was 6.5.

It is seen clearly from Figs. 3 and 4 (plotted from Table I) how sensitive the fluorescence of jus is to small additions of the unsaponifiable matter from cod-liver oil. Jus is an animal fat and it is probable that it contains a small amount of vitamin A. Though the fluorescence of the sample of jus used was quite considerable, it is possible that it was due entirely to the small amount of vitamin A present, for it seems probable that, if all the vitamin A were withdrawn, the fluorescence would sink nearly to zero. A sample of jus that had become rancid gave the fluorescence:

shade = 6,

quality = 8 (R), 17 (G), 75 (B).

When examined in the melted state, it appeared to be practically non-fluorescent.

The actual composition of the emitted light from jus is changed very considerably by the addition of unsaponifiable matter up to 0.66 Lovibond

blue units (calc.), but it is seen that very little change takes place on further addition, until the effect of the colouring matter of the unsaponifiable matter becomes apparent.

Hardened coconut oil has a bright blue fluorescence, and this exerts a strong effect on the fluorescence of the added unsaponifiable matter. Though the fluorescence of hardened coconut oil is very bright, if examined in the liquid condition, it is seen to extend through a deep zone, and thus a sufficient excess of unsaponifiable matter from cod-liver oil is able to cut out most of the blue fluorescence, and the quality becomes similar to that of jus with unsaponifiable matter.

### III. THE FLUORESCENCE OF BUTTER, AND THE EFFECT OF THE ADDITION OF UNSAPONIFIABLE MATTER FROM COD-LIVER OIL ON THE FLUORESCENCE OF MARGARINE.

The fluorescence of butter is yellow, varying somewhat with different samples. It is due almost entirely to the fat, and the separation of the aqueous phase does not make a great difference, as shown in Table II.

Table II. *Fluorescence readings on butters, butter-fats and margarines.*

		Shade	Quality		
			Red	Green	Blue
Irish butter	...	32	27	44	29
Danish butter I	...	34	24	41	35
Danish butter II	...	30	22	37	41
Irish butter-fat	...	31	31	46	23
New Zealand butter-fat	...	31	27	48	25
Danish butter-fat I	...	30	25	42	33
Danish butter-fat II	...	29	22	44	34
Margarine I	...	24	10	24	66
Margarine II	...	35	9	22	69

Table II also shows the fluorescence of two samples of margarine. Both of these gave blue fluorescences quite different from the fluorescence of butter, but one was considerably brighter than the other. The brighter sample contained vegetable fats only, but the fat of the duller sample contained 35 % oleo.

For comparison with Table II it may be of interest to give the colour readings on some of the same samples, *i.e.* when the samples are illuminated by white light from a standard source instead of by ultra-violet light (Table III).

Table III. *Colour readings on butters, butter-fats and margarines.*

		Shade	Quality		
			Red	Green	Blue
Irish butter	...	70	45	37	18
Danish butter I	...	65	43	37	20
New Zealand butter-fat	...	45	51	40	9
Danish butter-fat I	...	58	47	40	13
Margarine I	...	67	45	38	17
Margarine II	...	67	45	37	18

An outstanding difference between butter and margarine is that butter contains considerable amounts of vitamins A and D. This deficiency of margarine may be made good by the addition of a sufficient amount of the unsaponifiable matter from cod-liver oil (the amount is calculated to give an antimony trichloride blue figure for the margarine of 0.27), and the addition produces a considerable change in the fluorescence. The margarines so treated can always be readily distinguished by means of filtered ultra-violet light from similar margarines without the vitamin fraction (Table IV).

Table IV. *Effect of the addition of unsaponifiable matter from cod-liver oil upon the fluorescence of margarine.*

	Shade	Quality		
		Red	Green	Blue
Margarine I ... ..	24	10	24	66
Margarine I v (i.e. margarine I with the addition of cod-liver oil unsap. to antimony trichloride blue figure of 0.27) ... ..	28	16	32	52
Margarine II ... ..	35	9	22	69
Margarine II v (i.e. margarine II with cod-liver oil unsap. as above) ... ..	41	13	26	61
Danish butter I ... ..	34	24	41	35

It is seen that the change effected by the addition of unsaponifiable matter is towards butter fluorescence. The fluorescence of margarine I with the unsaponifiable matter is definitely yellowish, but as is readily seen in the analysis, it does not equal the yellowness of butter fluorescence. As more and more unsaponifiable matter is added, the fluorescence becomes brighter and more nearly yellow, but about five times the amount necessary to bring margarine to the same vitamin A content as butter must be added before the fluorescence approximates in quality to that of butter. It appears probable that this is due in part to the masking effect of the blue fluorescence of the vegetable fats; for the margarine I v (with 35 % oleo in the fat) approaches butter in its fluorescence much more closely than does the margarine II v (containing only vegetable fats). This, however, does not furnish a complete explanation, for a margarine made entirely with animal fat, and with the normal amount of cod-liver oil unsaponifiable matter added, is still not a match in fluorescence for butter.

Two differences between butter and ordinary margarine have so far been considered—the comparative richness of butter in vitamins, and the presence in margarine of certain vegetable fats—and both have been shown to be concerned in accounting for the difference observed between the fluorescences of butter and margarine. The only other factor that would seem likely to be of importance is a possible difference between the colouring matters present. The mixture of colouring matters usually added to margarine is non-fluorescent, and when an excess of it is added, it is seen that the fluorescence is very much depressed in brightness. Butter frequently contains added colouring matter but a large proportion of its colour, at least in spring and summer, is derived

from the pigments in the cow's food, and it might be expected that these pigments would be fluorescent. Moreover, anatto, the colouring matter commonly added to butter, is fluorescent. Margarine normally contains only a small proportion of natural colouring matter, derived from the beef-fat and the vegetable oils. Red palm oil is slightly fluorescent and very strongly coloured, and its pigment is closely related to the pigment in butter. In some countries it has been used for colouring margarine. 0.8 % of red palm oil added to oleo brings the colour to that of Danish butter-fat, and when unsaponifiable matter from cod-liver oil to the vitamin potency of butter-fat has been added, the fluorescence also is a match for that of Danish butter-fat (Table V).

Table V. *Effect of pigments on the fluorescence of fats.*

	Shade	Quality		
		Red	Green	Blue
Jus ... ..	29	14	33	53
Jus + 0.8 % red palm oil ... ..	20	22	43	35
Jus + 0.8 % red palm oil + unsap. to vitamin potency of butter-fat ... ..	25	26	42	32
Danish butter-fat I ... ..	30	25	42	33
Danish butter-fat II ... ..	29	22	44	34
Oleo + 0.8 % red palm oil + unsap. ... ..	31	24	43	33
Jus + 1.6 % red palm oil ... ..	16	27	45	28
Jus + 1.6 % red palm oil + unsap. ... ..	24	29	47	24
New Zealand butter-fat ... ..	31	27	48	25
Irish butter-fat ... ..	31	31	46	23
Oleo ... ..	44	11	34	55
Oleo + synthetic butter colour ... ..	14	15	34	51

It is observed that the addition of red palm oil to jus causes the blue component in the analysis to drop, and the red and green components to increase. At the same time the intensity of the fluorescence (shade) is depressed somewhat. The addition of unsaponifiable matter to the coloured fat increases the intensity of the fluorescence again, and further diminishes the blue component. The quality of the fluorescence from jus with unsaponifiable matter and 0.8 % red palm oil is a match for that of Danish butter-fat, and doubling the amount of pigment brings the quality to a match with that of New Zealand or Irish butter-fat. The shade figure for jus with unsaponifiable matter and 0.8 % red palm oil is lower than the shade figure for oleo with the same additions, but this is to be attributed to the greater opacity of jus. The greater opacity to ordinary light is obvious. The greater opacity to fluorescence-exciting rays can be demonstrated by shielding a crystal of uranium nitrate from the direct rays of the lamp by a thin layer of jus or oleo in a Petri dish. The crystal of uranium nitrate fluoresces much more strongly under the screen of oleo than under the screen of jus of the same thickness.

The samples that respectively match Danish butter-fat and New Zealand or Irish butter-fat in the quality of their fluorescence, also match them in the actual colour by ordinary illumination (Table VI). Here, as would be expected, the greater opacity of the jus increases the brightness by reflected light.



Table VI. *Actual colour by reflected light of samples that match in fluorescence.*

	Shade	Quality		
		Red	Green	Blue
Danish butter-fat I ... ..	58	47	40	13
Oleo + 0.8 % red palm oil + unsap. to vitamin potency of butter-fat ... ..	57	48	41	11
Jus + 0.8 % red palm oil + unsap. ... ..	69	47	39	14
New Zealand butter-fat ... ..	45	51	40	9
Jus + 1.6 % red palm oil + unsap. ... ..	53	52	39	9

## SUMMARY.

1. A method has been devised by which the actual brightness of the fluorescence of a solid fat, illuminated by ultra-violet light filtered practically free from visible light, may be determined, and the colour expressed in terms of three additive primaries: red, green and blue.

2. There is present in the unsaponifiable matter from cod-liver oil a brightly fluorescent substance. Curves and tables are given showing the effect of the addition of unsaponifiable matter from cod-liver oil on the fluorescence of two fats, one already slightly fluorescent (jus), and the other brightly fluorescent (hardened coconut oil).

3. The fluorescence of butter or butter-fat is yellow in colour, but the normal fluorescence of margarine is blue. This difference cannot be accounted for solely by the known differences in vitamin content.

4. The blue fluorescence of margarine can be modified as follows:

(a) by varying the fat mixture: certain vegetable fats fluoresce bright blue, while the fluorescence of oleo and jus is pale greenish;

(b) by the addition of unsaponifiable matter from cod-liver oil: the first small additions markedly increase the brightness and diminish the blueness of the fluorescence;

(c) by varying the nature of the pigment present: some pigments depress the fluorescence more markedly than others.

5. A sample of oleo coloured with sufficient red palm oil to match it with butter-fat, and with sufficient unsaponifiable matter from cod-liver oil to bring it up to butter-fat in vitamin A potency, exactly matches butter-fat in fluorescence.

## REFERENCES.

- Carr and Price (1926). *Biochem. J.* **20**, 497.  
 Gerngross and Schulz (1927). *Chem. Ztg.* **51**, 501.  
 Guild (1927). *Trans. Opt. Soc.* **27**, 106.  
 Peacock (1926). *Lancet*, ii, 328.  
 Popp (1927). *Z. Unters. Lebensmittel.*, **52**, 165.  
 Volmar (1927). *J. Pharm. Chim.* **5**, 435.

# CLXXXIX. NOTE ON THE ISOLATION OF MESACONIC ACID FROM CABBAGE LEAVES.

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In a paper published by the author some years ago [1923] in conjunction with Prof. Schryver, mention was made of the fact that mesaconic acid had been obtained from among the products extracted from green leaves (cabbage). As it appears that the presence of this substance among naturally occurring products had not previously been reported, it is thought that a rather more detailed account of its discovery might be of interest.

As stated in the original paper, the object of the research was, mainly, to examine the simpler nitrogenous constituents of green leaves, this being the natural extension of the earlier work of Chibnall and Schryver on the leaf-proteins.

The method adopted may be summarised briefly as follows.

90 kg. of fresh leaves were minced, the ether-water extract obtained, and the protein therein flocculated [cf. Chibnall and Schryver, 1921]. The clear solution was treated with alcohol (2 volumes of 95 % alcohol), and the white flocculent precipitate produced was removed; this is termed the "organic phosphate precipitate." After evaporation of the alcohol, the basic substances were precipitated by the usual phosphotungstic acid method. From the filtrate, sulphuric acid and excess of phosphotungstic acid were removed; the filtrate was then saturated with baryta and the barium salts of organic acids present were precipitated by the addition of excess of 95 % alcohol [cf. Dakin, 1921]. This precipitate is designated the "dicarboxylate fraction," consisting as it does mainly of the barium salts of dicarboxylic acids, nitrogenous and non-nitrogenous. It was from the "organic phosphate" and "dicarboxylate" fractions that mesaconic acid was isolated, the greater part being found in the latter.

The barium dicarboxylates were decomposed quantitatively by means of sulphuric acid, the amount necessary having been determined gravimetrically. The filtrate and washings were evaporated to a thick syrup; this was extracted repeatedly with alcohol, whereby the simple organic acids present were separated roughly from the nitrogenous dicarboxylic acids. The alcoholic liquid was evaporated to a thin syrup, and extracted with ether; on evapo-

rating, the ethereal extract partly crystallised. After removing the crystals, the residue was taken up in water, neutralised by the addition of calcium hydroxide, and the calcium salts were precipitated with alcohol. From the calcium salts thus formed, a second crop of crystals of the free acid was obtained, similar to those first separated. The substance was recrystallised from water, giving small colourless prisms, moderately soluble in cold water, readily soluble in hot water and alcohol, but sparingly soluble in ether. The total yield of the recrystallised product was 5.0 g.; M.P. 201°.

An elementary analysis revealed only the presence of carbon, hydrogen and oxygen. The substance was strongly acid, its equivalent, determined by titration with standard alkali, being 65.0. An estimation of the molecular weight by the cryoscopic method in aqueous solution gave a result of 124. The substance was therefore a dibasic acid. Permanganate was rapidly decolorised by the substance, as was also bromine water on warming slightly.

The above properties indicated that the substance was an unsaturated dicarboxylic acid, with a molecular weight of 130. Mesaconic acid,  $C_5H_4(COOH)_2$ , fulfils these requirements, and has M.P. 202°. The bromine addition product was prepared, and recrystallised from water, being then found to have M.P. 204°, with decomposition, agreeing with the M.P. of the *mesadibromomethylsuccinic* acid obtained on bromination of mesaconic acid. A mixed melting point of the original acid with a sample of pure mesaconic acid had M.P. 201–2°. The acid isolated was therefore certainly mesaconic acid.

As stated above, a further small amount (1 g.) of the acid was obtained from the "organic phosphate" fraction. This fraction consists mainly of the calcium and potassium salts of various inorganic and organic acids, and the mesaconic acid is apparently present as a calcium salt. It is liberated by treatment with dilute sulphuric acid, and is then extracted by alcohol, crystallising out when the alcoholic liquid is mixed with ether and allowed to stand. The total yield of mesaconic acid obtained in the two portions (6 g. from 90 kg. of leaves), is not to be regarded as a measure of the total amount present, as the methods of separation were by no means quantitative.

As far as can be ascertained, mesaconic acid has not previously been met with as a naturally occurring product. It is possibly connected with citric acid, which is a frequent constituent of plants, and was in fact isolated from the "dicarboxylate" fraction in the course of this work. Loss of water from citric acid gives aconitic acid, which is present as the calcium salt in the sugar-cane, sugar-beet, and specially in the leaves of *Adonis vernalis*. This acid is known to pass, by loss of carbon dioxide, into citraconic acid, the optical isomer of mesaconic acid. The change from citraconic acid to mesaconic acid is not easy of explanation; it seems certain, however, that mesaconic acid is present as such in the leaf, and is not an artefact. This view is supported by its isolation from the "organic phosphate" fraction, when only mild reagents, not usually regarded as capable of effecting any intramolecular change, were brought into contact with the acid.

The work on the constituents of green leaves, of which the investigation here described formed a small section, was carried out under the direction of Prof. S. B. Schryver, F.R.S.

## REFERENCES.

- Buston and Schryver (1923). *Biochem. J.* **17**, 470.  
Chibnall and Schryver (1921). *Biochem. J.* **15**, 60.  
Dakin (1921). *J. Biol. Chem.* **44**, 499.

# CXC. A STUDY OF THE OESTRUS-PRODUCING HORMONE: ITS PREPARATION AND STANDARDISATION IN A WATER-SOLUBLE FORM.

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*(Received November 5th, 1928.)*

SINCE very full reviews of the literature concerning the oestrus-producing hormone have recently been published [Allen and Doisy, 1927; Parkes, 1928], it is not proposed to enter into a detailed historical summary here, and only those papers particularly concerning the aspect at present under consideration are briefly reviewed. It is proposed to describe experiments leading to a method for the preparation of the oestrus-producing hormone in a highly active, water-soluble form and its properties in this state. That such a product would have considerable advantages for clinical and experimental purposes over the existing oily material, requires but little comment. Whilst Ralls, Jordan and Doisy [1926] state that they can obtain from liquor folliculi an oily preparation containing 1 unit in 0.02 mg. of solid material, experiments here have not succeeded in reducing the unit by this method to below 0.5 mg., but this discrepancy may be due to differences in the technique of standardisation which are discussed later.

There have been many claims to the preparation of a water-soluble product. It will only be necessary to refer here to the main workers. Zondek and Brahn [1925] were among the first to describe a water-soluble extract, and in the same year Loewe [1925] obtained an active aqueous solution. Glimm and Wadehn [1926], and Frank *et al.* [1926] also describe similar preparations. By far the most important claim is that put forward by Laqueur and his co-workers [1925, 1926, 1927]. Although Prof. Laqueur has sent us water-soluble material of high activity, we have not succeeded in obtaining satisfactory results by means of the methods published by him and his associates. Whilst we have obtained active material by his method, the yields have been very small and inconstant. In 1927 [Dickens, Dodds and Brinkworth, 1927] a method was also published by us, but as has already been pointed out [Dodds, 1928], this has not proved satisfactory.

Since all these methods have been adversely criticised it was decided to attack the problem *de novo*. As the work progressed two separate problems arose; firstly the question of preparation, and secondly the method of standardisation.

In all these experiments placenta has been used as the starting material. The first attempts to produce a water-soluble preparation were made by acid

digestion of the placenta. Whilst an apparent aqueous solution containing the active principle could be obtained by this procedure, the crude material produced local abscesses in the animals. Although the final product had the appearance of an aqueous solution, the active principle could be extracted by means of ether or butyl alcohol and was associated with material of fatty nature; a colloidal suspension of fats had been produced. It was evident that a method had to be adopted involving the destruction of the fats. This was accomplished by the use of barium hydroxide in place of acid for the initial digestion of the placenta; after extraction of the unsaponified fat, this was resaponified with the barium hydroxide and a clear aqueous solution was obtained after removal of the soaps. The activity, however, had almost completely disappeared, and it was only possible to produce a pro-oestrus response in the animals by injecting this material according to the usual single dose method. It appeared from these experiments, therefore, that the potency was destroyed by this treatment.

Up to this point, except in the earliest experiments, standardisations had been carried out by the method of Coward and Burn [1927]: a number of ovariectomised animals were injected with a single dose of the material and the response was studied by vaginal smears, the results being calculated on the percentage of rats giving full oestrus. By this method the resaponified material gave no oestrus response. At this stage in the investigations an aqueous specimen of material was received from Prof. Laqueur which, when tested by the method of standardisation already referred to, was also found to be inactive, but when injected in a series of small doses according to the method described by Laqueur and his co-workers, proved to be highly potent. A re-standardisation on these lines of the aqueous material made by the barium hydroxide method resulted in a full oestrus response in a large percentage of animals, in contrast to an entirely negative result when injected in a single dose. With this knowledge it was possible to evolve a process for the preparation of water-soluble material of high potency.

#### EXPERIMENTAL.

##### *Standardisation.*

The following table gives the results of a single injection as contrasted with the response obtained when the quantity of the dose is divided into six equal portions.

Table I.

Batch No.	No. of rats	Dose	No. of rats showing oestrus	Result %
215	10	1 dose of 1 cc.	1	10
258	7	1 dose of 1 cc.	0	0
215	15	6 doses of 0.2 cc. (interval as under "method")	13	86
258	10	6 doses of 0.2 cc.	6	60
278	20	6 doses of 0.2 cc.	11	55
278	20	4 doses of 0.3 cc. in 12 hours	0	0

This demonstrates that the single injection method was not applicable to water-soluble material and, moreover, it fully confirms the claims of Laqueur and his co-workers as to the activity of water-soluble material when injected in multiple doses. In addition to dividing the dose, the time interval between the injections is very important. Thus, six doses distributed as described give a positive result, whereas the same total quantity, if given by the method described by Allen *et al.* [1924] is inactive. Results such as these led to the adoption of a method combining the multiple dose technique of Laqueur with the "statistical" method of Coward and Burn.

*Method.* All standardisations have been performed upon ovariectomised rats. When a weight of 140 g. is reached the ovaries are removed and vaginal smears are taken for 2 weeks. If no signs of cyclical activity are observed, the animals are then put into use. Six doses of 0.2 cc. each are injected into the animals, the preparations being diluted to contain approximately 1 unit in 1.2 cc. The injections are made as follows: first day, 9 a.m. and 6 p.m.; second day, 9 a.m., 3 p.m. and 9 p.m.; third day, 9 a.m. only. Smears are taken at 9 a.m. on the first and second days; at 9 a.m. and 6 p.m. on the third day; and again at 9 a.m. and 6 p.m. on the fourth day. It is rare for a rat to attain oestrus after the evening of the fourth day, and often signs of a return to the dioestrus condition are already present in the sixth smear. A minimum of 20 rats and, where possible, 100 rats are used for each batch. Smears have been stained with haematoxylin and eosin, since this enables a permanent record to be kept of the response elicited by various preparations.

*Interpretation.* One unit of activity is that quantity of material required to produce a full oestrus response in 50 % of the rats used (Coward and Burn). Oestrus has been taken to be present when cornified cells only are shown in the vaginal smear.

Table II.

Batch No.	No. of rats	No. showing oestrus	Calculated unit cc.	Difference between duplicates cc.
260	20	9	1.33	
	30	16	1.12	0.21
279	20	13	0.92	
	20	14	0.86	0.06
281	19	14	0.81	
	20	9	1.33	0.52
290	50	26	1.15	
	52	29	1.08	0.07
290 a	40	17	1.40	
	36	10	2.16	0.76
292	20	7	1.71	
	21	14	0.90	0.81

In view of the striking differences between the action of the hormone in oily and water-soluble form, we decided to construct a curve connecting the percentage of oestrus response and the number of rats used, similar to that recorded by Coward and Burn. Owing to the large number of determinations necessary in order to establish such a curve, our experiments upon this point

are at present incomplete. That the method described above is far from satisfactory is shown by a series of duplicate standardisations taken at random from our results.

Table II shows that even with the precautions of the method quoted, the unit may vary by as much as 5 to 50 %. Although Coward and Burn pointed out a year ago that there is a considerable possible error in the use of 25 rats, and the above experiments fully bear out their views, some writers do not even mention the number of animals used for a single test, and assume for their standardisation what must now be regarded as an entirely fictitious accuracy. The statistical method is much superior to the diminishing dose technique, and until further experiments have produced a better definition we have adopted the 50 % response as a unit.

As previously shown [Dickens, Dodds and Wright, 1925] the crude material produced by alcoholic extraction from ovaries considerably depressed the blood-pressure. The water-soluble material obtained by the method described below is, however, entirely without action on the blood-pressure, even in doses containing 50 units, when injected into a cat under chloralose and ether anaesthesia.

The effect on the uterus has also been examined by the method of Dale and Burn, and it was found that the addition of the extract in doses of 50 units to the uterine bath (125 cc.) produced a series of very small and slow contractions and relaxations. Further work is being done on this point.

#### *Preparation.*

*Acid digestion of the placenta.* Digestion of the placenta with acid [Dickens, Dodds and Brinkworth, 1927] results in nearly all the placental material entering into solution. The great stability of the hormone was shown by digesting minced placenta (1300 g.) with 650 cc. of 5 % hydrochloric acid in an autoclave at 150° with constant stirring for 7½ hours. The resulting suspension, when cold, was centrifuged, and the reaction of the clear, dark-coloured fluid (1770 cc., precipitate discarded) was adjusted to  $p_H$  4, when an isoelectric precipitate appeared. This was dissolved in a minimum of 0.1 *N* alkali and was made up to 1000 cc. and 1 cc. of this slightly turbid solution produced oestrus.

This extract was very crude and the adsorption of activity on to the precipitate was not constant. Extraction of the activity from the acid digest was attempted with butyl alcohol [Dickens, Dodds and Brinkworth, 1927]. 2.5 kg. of minced placenta and enough concentrated hydrochloric acid to bring the  $p_H$  to 1.8 were heated in an autoclave at 150° with stirring for 1½ hours, and were allowed to cool. The mixture was filtered (filtrate 2430 cc.,  $p_H$  3.0) and was extracted with butyl alcohol for 10 hours in a constant-extraction apparatus. The dark-coloured extract deposited a colourless crystalline precipitate (amino-acids?) which was removed, and a coagulum separating in the aqueous part was extracted with butyl alcohol. The combined butyl alcohol extracts were evaporated *in vacuo* to a dark-coloured



residue completely soluble in hot water. The reaction was strongly acid, and on neutralising a turbidity appeared. The volume of this crude extract was 800 cc., and 0.5 cc. caused a definite oestrus response. The active material was still associated with fatty substance as was shown by ether extraction, evaporation of the ether extract yielding a suspension (*A*) of fat globules in water (480 cc.) of which 0.5 cc. produced a definite oestrus response. Centrifuging separated the fat droplets and standardisation showed that this layer carried with it the activity. It was therefore concluded that in order to obtain an aqueous preparation, preliminary destruction or removal of the fats was necessary. The simplest method of bringing this about appeared to be saponification.

*Saponification.* It was proved that the hormone in the fat-containing preparations is stable to the action of boiling alkali. Thus the suspension (*A*) (see above), was divided into four specimens. To the first 0.1 *N* sodium hydroxide was added until the  $p_H$  was between 8 and 9, to the second an equal volume of normal sodium hydroxide solution was added, and to the third an equal volume of 20 % potassium hydroxide. The fourth specimen served as a control. All the four specimens were heated in a boiling water-bath for 3 hours with vigorous shaking at intervals. Each was cooled, acidified with hydrochloric acid, and extracted with ether; the ethereal extracts were washed with a little water and evaporated. The residues, containing fatty acids, were emulsified in dilute sodium carbonate solution (volume of each specimen equal to half the initial volume of (*A*) used), and doses of 1 cc. were given (*i.e.* four times the dose of (*A*)). Each of the four specimens produced oestrus, showing that the active material had survived this treatment.

In order to determine the best conditions for saponification with minimum loss of activity, a quantity of fatty material was prepared from placenta by a modification of Allen and Doisy's method [Dickens, Dodds and Wright, 1925]. After freezing out most of the fats from the acetone solution of the final product, and precipitating the cholesterol by digitonin, 9.39 kg. of placenta gave 2.59 g. of clear oily substance (No. 217), of which a solution in olive oil containing 25 mg. per cc. was prepared for injection, the results of which are tabulated below for comparison with those obtained after saponification<sup>1</sup>.

Table III.

Dose in mg.	No. of rats	Results
2.5	20	2 E, 5 E-, 11 P
5.0	10	5 E, 3 E-, 1 P
10.0	6	2 E, 2 E-, 2 P
30.0	6	5 E, 1 E-

*Note.* E denotes complete cornification; E- denotes a similar smear but containing a few nucleated epithelial cells; and P denotes a definite pro-oestrus smear.

For the following experiments a solution of 2.086 g. of No. 217 in 50 cc. absolute alcohol (41.7 mg. per cc.) was prepared.

<sup>1</sup> The experiments described here were performed before the results of Coward and Burn's work on the standardisation of the hormone were known to us.

*Determination of saponification value.* The solution (5 cc.) was boiled under reflux for 2 hours with 0.0909 *N* alcoholic potassium hydroxide (25 cc.), cooled and titrated against 0.0988 *N* hydrochloric acid using phenolphthalein (required 15.3 cc.). The saponification value was, therefore, 205. The contents of the flask were evaporated to a clear, reddish-brown, aqueous solution (5 cc.), which was neutral to phenolphthalein, and was injected in single doses of 0.5 cc. into rats (no. of animals 7; result, 5 E, 2 P).

*Saponification by the calculated amount of alkali.* In the above experiment an excess of alcoholic potassium hydroxide was used. In a similar experiment using the calculated amount (8.4 cc. 0.0909 *N*) of potassium hydroxide in alcohol after boiling under reflux for 2 hours, the reaction was neutral to phenolphthalein, and the solution was evaporated on the water-bath to a very small volume and made up to 5 cc. with water. The solution was very faintly opalescent. A dose of 0.15 cc. was injected into rats (8 animals; result, 1 E, 3 E-, 4 P). Saponification of 5 cc. by boiling for 1 hour with sodium in alcohol (4.5 cc. of a solution of 0.40 g. sodium in 100 cc. absolute alcohol) followed by evaporation and neutralisation with 0.5 cc. 0.1 *N* hydrochloric acid, gave an aqueous preparation (5 cc.) which set to a jelly on allowing to stand for some days. Doses of 0.15 cc. each were injected into rats (7 animals; result, 1 E, 5 P).

*Removal of soaps.* These experiments show qualitatively that the fats can be saponified without complete destruction of the hormone. If alkalis are used, the resulting product contains the corresponding soaps in aqueous solution. Removal of these soaps by replacing the alkali metals by barium was attempted in two ways: (1) by direct saponification of the oily material by boiling with barium hydroxide, and (2) by addition of barium chloride to the solution of potassium soaps obtained from the oily material as above.

(1) The stock solution (No. 217, 5 cc.) was boiled for 2 hours under reflux with the addition of the calculated amount (3.8 cc. 0.2 *N*) of barium hydroxide solution. The neutral alcoholic solution was evaporated, a little water being added, and the precipitate of barium soaps (*B*) was filtered off. The filtrate was freed from barium by cautious precipitation with 1 % sulphuric acid solution and filtration from the precipitate of barium sulphate. The clear yellow filtrate was evaporated to 5 cc. and contained 5.4 mg. of solid matter (dried at 110°) per cc. Doses of 0.15 cc. proved to be inactive (2 P only). A similar experiment in which twice the calculated quantity of barium hydroxide was used gave a similar result (P only being obtained even with three times the dose given above). The barium soaps (*B*) were taken up in hot 50 % alcohol and 1 % sulphuric acid was added until the reaction was strongly acid. After half an hour on the boiling water-bath, barium hydroxide solution was added until the acidity was reduced to a weakly acid reaction, the precipitate of barium sulphate was filtered off, and the alcohol removed from the filtrate by distillation. The fatty acids which separated were again emulsified by addition of dilute sodium bicarbonate solution, the final volume being 5.5 cc. Doses of 0.3 cc. were inactive. Thus the activity of the extract

could not be recovered from the precipitate of barium soaps and was apparently destroyed.

(2) Attempts to trace the exact stage at which the loss occurred were made by converting the fats into the potassium soaps and precipitating by addition of barium chloride solution. 23.5 cc. of the stock solution in alcohol (No. 217) were saponified as before by boiling with 0.1 *N* alcoholic potassium hydroxide solution (40 cc.) for 2 hours, the reaction remaining alkaline throughout. The slight excess of alkali was neutralised by 0.1 *N* hydrochloric acid (1.5 cc.) and the alcohol was evaporated. The resulting aqueous solution (23.5 cc.) was standardised (10 rats; single dose of 0.2 cc.; result, 5 E, 3 P). 5 cc. of this solution were diluted to 10 cc. and heated on the water-bath, and a slight excess of barium chloride solution (9.4 cc. approximately 0.1 *N*) was added. The resulting precipitate of barium soaps was centrifuged, and the clear supernatant fluid freed from the excess of barium by sulphuric acid. The final volume was 5 cc.; when injected into 6 rats in single doses of 0.25 cc. it was completely inactive.

These results were obtained only with fairly pure fatty material. We found in similar experiments in which crude oily preparations made from placenta by the usual alcohol process, with removal of the lipins but without subsequent purification by freezing and by digitonin, were boiled with barium hydroxide, much of the activity passed into the aqueous filtrate. In one experiment the oily material (10.5 g., which in doses of 75 mg. to each of 10 rats gave 9 oestrus responses) was dissolved in hot aqueous alcohol (160 cc. of 70 % alcohol) containing 5 g. of crystalline barium hydroxide in solution and the mixture was boiled under reflux for 2 hours. The reaction not being markedly alkaline, a further 2 g. of barium hydroxide crystals dissolved in 10 cc. of water were added, and boiling was continued for another half hour. A precipitate had formed, and distillation of the alcohol caused a further amount to collect. The residue was cooled on ice and centrifuged; the clear aqueous supernatant fluid when heated, became turbid, and this precipitate was also removed by filtration. Removal of the barium by sulphuric acid gave a faintly opalescent filtrate (40 cc.) (10 rats; 1 cc. each; result, 5 E, 3 E-, 3 P). This result suggests that the hormone was carried into the final solution in this experiment adsorbed on to a colloid (? cholesterol). That the final solution contained lipid material was shown by treatment in a continuous extraction apparatus with ether, evaporation of the ether yielding a trace of oily material which could not be redissolved in water, but which, on emulsification with dilute sodium bicarbonate solution and injection into rats, evoked an oestrus response. This method of preparation from the fatty material we regarded, at the time of these experiments, as unsuitable for the preparation of aqueous solutions of the hormone, since it failed with purified fatty material. As already described under "Standardisation," material of this type was found to be active when given to rats by the multiple injection method. It seemed probable that in the experiments in which soaps remained present in the final material

after saponification (those in which potassium hydroxide or sodium ethoxide was used) the presence of these substances caused the material to act like the original oily preparations; whereas the use of barium, and consequent removal of the insoluble barium soaps, produced a different type of substance, in aqueous solution, inactive in one dose, but giving a positive response when administered by multiple doses. That it is possible to reverse this effect is shown by taking some of the water-soluble product, the preparation of which is described later, and adding potassium oleate solution to it before injection. In one experiment, the material in a single dose of 0.15 cc. injected into 10 rats gave an entirely negative response; but injection into 7 rats of a single dose of 0.1 cc. mixed before injection with 0.1 cc. of a solution of potassium oleate, produced 1 oestrus and 6 pro-oestrus responses; whilst the same material without potassium oleate, given to 15 rats as 6 injections, the total amount of which for each animal was equal to 0.08 cc., caused 13 of the rats to show an oestrus response.

*Alkaline digestion of the placenta.* These experiments suggested the use of alkali instead of acid for the digestion of the placenta, the object being the early elimination of as much of the saponifiable fats as possible. In a typical experiment 500 g. of the minced placenta together with an equal volume of water and 200 g. of crystalline barium hydroxide were heated on the steam-bath, the temperature inside the flask being about 85°, for 9 hours. After cooling and filtering (precipitate rejected), the filtrate, which was clear and light in colour, was freed from barium by sulphuric acid (alcohol extraction of the precipitate of barium sulphate yielded no active material). The clear aqueous solution so obtained (300 cc., filtrate "C") was active in single doses of 1 cc., and the potency seemed to be in solution since it could be filtered with practically no loss of potency. Investigations on the concentrations of barium hydroxide, time and temperature, showed that periods of boiling, even up to 8 hours with an equal volume of 20 % barium hydroxide, did not destroy the activity; whilst digestion for half an hour at the boiling-point was shown to be insufficient, since an alcoholic extract of the precipitate filtered off after digestion, and also of the precipitate of barium sulphate, gave active material in these experiments, and the final product was without activity—possibly due to insufficient saponification. Very slow digestion at 70° for 18 hours presented no advantage over brisk boiling for 2 hours in a steam-jacketed still under reflux with an equal volume of 20 % barium hydroxide solution, and this was the method finally adopted. Digestion of the placenta with alcoholic barium hydroxide has also been found to be suitable—boiling for 1 hour with two volumes of alcohol and barium hydroxide crystals to 10 % of the total volume effects very complete digestion, and on filtering from the precipitate and evaporating the alcohol a concentrated solution remains containing the activity. We have used mainly aqueous digestion in the preparation of our extracts.

*Purification by alcohol and ether.* The crude product obtained in this way

contained a large quantity of protein fission products. It gave a copious precipitate with picric acid, a small precipitate on saturation with ammonium sulphate, no precipitate with trichloroacetic acid, and a pink biuret reaction. It was toxic, producing a severe local reaction. In order to purify it and to remove the protein digestion products, precipitation by alcohol and ether was tried: the filtrate "C" obtained in the experiment described above (300 cc.) was evaporated to 150 cc. ( $p_H$  2.8) and 10 volumes of absolute alcohol were added gradually with stirring. The alcohol was filtered from the resultant sticky precipitate of peptone (rejected, being found inactive and toxic). Removal of the alcohol left a viscous residue which was dissolved in water (52.5 cc.), single doses of 0.25 to 0.5 cc. being found active. The solution still gave a pink biuret reaction and a precipitate with picric acid. These properties were largely lost on reprecipitation from 95 % alcohol. The white precipitate forming was found to contain little or no activity. The alcoholic solution was added to twice its volume of ether when a brownish sticky precipitate collected, and this also was found to be without activity. The ethereal solution was evaporated, giving a small trace of yellow oil which, when suspended in water (75 cc.), had an acid reaction and was, therefore, neutralised by a small amount of sodium hydroxide. Doses of 0.5 cc. showed definite activity. (The use of acetone instead of alcohol and ether led to a similar product.)

This preparation still contained a small amount of fatty substance which was removed by boiling again with barium hydroxide. 20 cc. were added to 10 cc. cold saturated aqueous solution of barium hydroxide and the mixture was boiled. Evil-smelling bases were evolved, and boiling was continued until their removal was complete ( $\frac{3}{4}$  hour). A precipitate had then settled and was removed from the solution by filtration, the barium was quantitatively removed from the filtrate by sulphuric acid and the volume made up to 20 cc. (Standardisation on 8 rats; dose 1 cc.; result, 1 E-, 4 P.) Trichloroacetic acid, copper sulphate, acid and alkali caused no precipitation, picric acid a very faint one, but the biuret and other protein reactions were still positive and a precipitate was obtained with tungstic, tannic, and phosphotungstic acids, and also with mercuric chloride. These precipitants were not suitable for purification, the active material being apparently divided between the precipitate and the liquid. (That a response was obtained by the single injection method in these experiments is to be attributed to the relatively crude nature of the final product.)

*Extraction with ether and with butyl alcohol.* In all experiments of this type the final product contained nearly all the protein disintegration products extracted by butyl alcohol. Alcohol and ether precipitation following the butyl alcohol extraction, besides involving the use of large quantities of these solvents, did not yield a product of sufficient purity. Direct ether extraction of the placental digest was insufficient, as shown by the large yield of active material obtained by subsequent extraction with butyl alcohol. Thus the filtrate obtained after digestion with barium hydroxide in the usual way

(17.1 litres, concentrated to 4.7 litres, from 10.1 kg. of placenta) was extracted in a shaking apparatus with ether (two extractions, each with 1.5 litres ether). The ether was distilled off and the light-coloured, oily residue was dissolved in alcohol, a trace of barium was removed by precipitation with sulphuric acid and the alcohol was evaporated from the filtrate, which was then made slightly alkaline and injected as an emulsion (volume 22.5 cc.; dose 0.1 cc. to each of 6 rats; result, 1 E-, 2 P). The aqueous layer after ether extraction was then extracted under the same conditions with butyl alcohol (two extractions, each with 1500 cc.), the butyl alcohol was evaporated and the large residue dissolved in about 1 litre of hot water, and this was shaken with ether (two extractions, each with 500 cc. ether). The ether extract was filtered, concentrated, and all traces of butyl alcohol were distilled off, and an equal volume of alcohol was added to the aqueous residue. Barium hydroxide solution (10 cc., 5 %) was added and the mixture was boiled under reflux for 2 hours. The resulting precipitate was filtered from the cold solution, the barium was removed by sulphuric acid, and the filtrate (75 cc.), a clear yellow solution, contained 15.6 mg. of solid matter per cc. It was standardised first in a single dose of 0.15 cc. (6 rats; result, 5 P) and later, after diluting 15 times, by injections of 6 doses of 0.2 cc. to each animal (total amount equivalent to 0.08 cc. of the undiluted material: 15 rats; result, 13 E, 1 E-, 1 P). The material contained in the aqueous layer after ether extraction, when concentrated, did not produce oestrus. This method, omitting the preliminary ether extraction of the barium hydroxide digest, has been repeated many times, and active material has consistently been obtained. The following table summarises the results from some typical batches (the multiple dose method of standardisation is used in this and in all the subsequent experiments).

Table IV.

Batch No.	Weight of placenta kg.	Final volume cc.	No. of rats	Approx. unit in cc. (undiluted)	Units per kg.	Solids (detd. at 110°)	
						per cc. mg.	per unit mg.
215	10.1	75	45	0.05	150	15.6	0.78
241	4.35	90	11	0.14	145	2.18	0.13
237	3.1	70	10	0.17	135	—	—
242	3.12	65	10	0.15	290	7.64	1.14
247	3.09	55	20	0.1	180	—	—

The material, though now without toxic and irritant properties, was still crude, as may be seen from the last column in the above table. It also contained a high percentage of nitrogen (for example, No. 241 had 0.335 mg. nitrogen per cc., or 0.047 mg. per unit) and contained substances giving a pink biuret reaction, and a faint but definite Millon reaction. Nessler's solution caused the formation of a cream-coloured precipitate; phosphotungstic acid also gave a voluminous precipitate. A portion (5 cc.), evaporated to dryness and extracted with chloroform, gave negative reactions for cholesterol. The substance is not precipitated by trichloroacetic acid or tungstic acid, nor by

95 % alcohol. Addition of chloroform to the alcoholic solution caused a white precipitate to separate. The properties of the solution suggested that it contained bases.

*Removal of basic impurities.* The ether extract (500 cc.) obtained as above from 4.83 kg. of placenta, after washing with water, was washed successively with (1) 10 cc. 0.1 *N* HCl, (2) 5 cc. 0.1 *N* HCl and water, and (3) 20 cc. water in two portions. The combined extracts, of which (1) and (2) were strongly acid in reaction, were warmed to remove ether, and a dilute solution of sodium sulphate was added to precipitate the small quantity of barium, which was removed; the filtrate was neutralised with 1 cc. of *N* sodium hydroxide and was found to be inactive. The nitrogen content was 0.168 mg. per cc., the total amount extracted being 11 mg. The ether solution was washed twice with water (the second washing gave no precipitate with silver nitrate) and was then evaporated. The pale yellow residue was dissolved in 50 % alcohol (50 cc.), 5 cc. of 5 % barium hydroxide were added and the mixture was boiled under reflux for 2 hours. The alcohol was distilled off and the aqueous residue was allowed to stand in the ice-chest overnight, after which the precipitate (*A*) was removed by centrifuging and the barium removed from the clear supernatant fluid by sulphuric acid. The resulting clear, pale yellow, aqueous solution (40 cc.) was combined with that obtained in a similar way by repeating the digestion of the precipitate (*A*) in 25 cc. of 50 % alcohol with 2 cc. of 5 % barium hydroxide for 1½ hours, and removing the soaps and excess of barium hydroxide. The combined volume was 65 cc. and a portion was diluted 24 times for standardisation. Six doses of 0.2 cc. were given to each of 20 rats, 10 of which showed an oestrus response; hence the unit was taken to be 0.05 cc. of the undiluted product. The total yield was 1300 units, or 270 per kg. of placenta. The solution contained 1.0 mg. per cc. of solid residue (dried at 110°) and analysis by the micro-Kjeldahl method (Pregl) showed 0.050 mg. N per cc.; thus the unit was 0.05 mg. and contained 0.0025 mg. N, the total nitrogen in the 65 cc. being 3.25 mg. and, therefore, the shaking with hydrochloric acid had removed 75 % of the total nitrogen contained in the ether extract. Further extractions with hydrochloric acid remove only a negligible quantity. The product, after purification in this way, no longer gave the biuret test, the Millon reaction was faint but positive, whilst phosphotungstic acid produced a very slight, or no precipitate. The colour given with the Folin phenol reagent was, in this experiment, approximately equal to that given by a solution containing 0.1 mg. of tyrosine per cc. (equivalent to 0.0004 mg. tyrosine-N per unit). In Table V, Nos. 258 to 279, the properties and yields obtained by this method in five typical batches are given.

*Remarks on the method.* Fresh placenta has been used in nearly all batches, but in a few cases in which the material was slightly putrescent the yields were considerably diminished. In one experiment when definitely putrid material was used, we obtained only 25 units per kg. of placenta. Three extractions with butyl alcohol are usually sufficient, though, as experiment No. 324 in Table V

Table V.

Batch No.	Wt. of placenta kg.	Final vol. cc.	Standn. no. of rats	Approx. unit cc.	Units per kg.	Solids (detd. at 110°)		Nitrogen	
						per cc. mg.	per unit mg.	per cc. mg.	per unit mg.
258	4.83	65	20	0.05	270	1.0	0.050	0.05	0.0025
260	8.01	82	50	0.050	205	0.52	0.026	0.016	0.0008
274	6.2	200	20	0.20	160	0.50	0.10	0.036	0.007
278	7.0	162	20	0.18	130	—	—	—	—
279	5.35	375	40	0.46	150	0.20	0.092	—	—
280	11.35	320	20	0.15	180	0.37	0.06	—	—
281	10.5	345	110	0.25	190	0.14	0.035	0.007	0.0017
282	11.05	120	20	0.071	210	1.18	0.084	—	—
284	9.65	100	20	0.05	200	—	—	—	—
285	15.98	135	20	0.067	125	0.56	0.069	0.04	0.0047
288	14.2	120	20	0.083	105	0.148	0.0123	—	—
295	33.9	227	20	0.052	125	0.28	0.018	0.0147	0.00094
304	32.4	225	19	0.08	90	0.30	0.025	0.013	0.0011
305	17.7	105	20	0.055	110	—	—	—	—
309	31.0	310	20	0.09	110	0.20	0.018	0.006	0.0006
321	6.45	90	20	0.066	210	1.12	0.07	0.11	0.007
324 a)	14.0	{ 150	20	0.07	{ 156	0.48	0.034	0.043	0.003
324 b)		{ 90	20	0.2	{ 33	0.64	0.12	0.060	0.012
328	15.0	130	20	0.06	150	0.40	0.024	0.026	0.0015
331	1.78	100	20	0.35	160	0.16	0.05	0.012	0.004
332	1.70	160	20	0.9	100	0.18	0.16	0.016	0.014
345	61.5	550	20	0.07	125	0.18	0.013	0.028	0.002
292	13.15	130	41	0.11	90	0.09	0.010	0.010	0.001
310	5.0	60	20	0.13	92	0.18	0.023	0.012	0.0016
311)	5.0	{ 100	20	0.38	{ 52	0.68	0.26	0.085	0.032
312)		{ 120	20	0.30	{ 80	0.36	0.108	0.033	0.010
313	16.0	160	20	0.15	65	0.18	0.027	0.015	0.0023

*Notes.*

No. 279. Alcoholic barium hydroxide used for digestion of placenta.

No. 321. Placenta had been frozen at  $-5^{\circ}$  for 15 days before use.

No. 324 a. First three extractions with butyl alcohol, of barium hydroxide filtrate, combined and worked up as usual.

No. 324 b. Second three extractions from above (making 6 in all) worked up separately from 324 a by the usual process.

Nos. 331 and 332. The same batch of placenta was divided into two portions, No. 331 was used at once, and No. 332 was stored for 5 days in an equal volume of 0.5 % solution of tricresol in water at room temperature before digestion with barium hydroxide as usual.

No. 292. Three ether extractions.

shows, the extraction is then not quite complete. Direct extraction with ether is much less convenient owing to the formation of emulsions and is also less efficient as in the following example: the placenta (10 kg.) was boiled in the usual way with barium hydroxide and the resulting filtrate was concentrated and exactly half was extracted with ether (three extractions separated in the centrifuge). The combined ethereal extract after evaporation was treated with barium hydroxide as usual and the final aqueous product was No. 311, containing about 260 units. The filtrate, which had been extracted as above with ether, was afterwards extracted with butyl alcohol and the extract treated in a similar way (No. 312, a further 400 units). The other half of the original barium hydroxide digest was extracted directly with butyl alcohol as usual and gave about 460 units when treated in the usual manner. These results are summarised at the end of Table V where the result of an experiment (No. 313)



is included in which a mixture of butyl alcohol (30 %) and ether (70 %) was used for extraction; this, however, was not so complete as when butyl alcohol alone was used. The use of specially purified butyl alcohol of very low nitrogen content does not increase the yield or degree of purity of the final product.

Whilst the washing with hydrochloric acid of the ether solution obtained from the evaporated butyl alcohol extract is not accompanied by any loss of potency, the use of alkali for this purpose results in a partition of the activity. In one experiment on this point, the ether extract resulting from 1.55 kg. of placenta (140 cc.) was shaken mechanically for  $\frac{1}{2}$  hour with 0.1 *N* sodium hydroxide in two portions of 100 cc. each. The ether was then washed with water and the combined washings and alkaline extracts were made fairly strongly acid with *N* sulphuric acid. This solution was then shaken with ether, and, after evaporation of the ether, the residue was taken up in 50 % alcohol, 10 cc. of 5 % barium hydroxide were added and the mixture was boiled under reflux for 2 hours. After removal of alcohol, barium soaps, and then excess of barium, the filtrate (107 cc.) contained 125 units (20 rats; 6 doses of 0.2 cc. each; result, 14 E; approximate unit 0.85 cc., containing 0.29 mg. solids and 0.0082 mg. N per unit). The ether extract, after the above washing with sodium hydroxide, was evaporated and put through the usual process. The final volume was 174 cc. containing 85 units (20 rats; 6 doses of 0.2 cc. each; result, 6 E; approximate unit 2.0 cc., containing 0.17 mg. solids and 0.0017 mg. N per unit). This is an example of the activity being carried with the sodium soaps, and appears to be an absorption phenomenon, numerous examples of which have occurred in connection with the hormone.

The next point requiring quantitative investigation was the saponification with barium hydroxide of the oily residue obtained on evaporation of the ether. In certain of the earlier experiments a considerable loss of activity occurred at this stage, and we have found that this was due, not to inactivation, but to adsorption of the active material on the precipitate of barium soaps, and was probably associated with incomplete saponification. If the precipitate of barium soaps was filtered off and again boiled with barium hydroxide solution under reflux, a further yield of active material was obtained in these cases. In our later experiments we have also preferred to use an aqueous medium for the saponification rather than an alcoholic one, since the danger of loss from this cause appears to be less in an aqueous suspension. Under these conditions this operation is accompanied by a small loss only, as the following experiment shows.

The ether extract obtained in the usual way from 14 kg. of placenta was divided into two equal portions and both were evaporated to small oily residues; one of these was dissolved in a very little alcohol and poured into water and evaporated to drive off most of the alcohol, giving a fine, stable suspension in water (126 cc.). Injection of this material (diluted 8 times for standardisation; 6 doses of 0.2 cc. each to 20 rats; unit 0.14 cc. (undiluted) containing 0.56 mg. of solids and 0.0035 mg. N per unit) showed that it

contained in all 880 units. The oil from the other half of the evaporated ether extract was also suspended in a similar volume of water, 10 cc. of 5 % barium hydroxide were added to the aqueous suspension, and the mixture was boiled under reflux for 1 hour. The slight precipitate which separated during boiling was removed by filtration and barium was precipitated from the clear filtrate by heating on the water-bath and cautiously adding 0.1 *N* sulphuric acid until the reaction was very slightly acid. The precipitate of barium sulphate was filtered off and was washed with a little hot water. The filtrate (145 cc., diluted 7 times for standardisation; 6 doses of 0.2 cc. to each of 19 rats; unit 0.19 cc. (undiluted); 0.026 mg. of solid material and 0.0017 mg. *N* per unit) contained in all 760 units. The loss in this experiment was within the experimental error of standardisation.

Great care is necessary in the removal of barium from the final product. In the above example the addition of an excess of 0.5 cc. 0.1 *N* sulphuric acid to the total volume increased the "solid" content (weight of the residue at 110°) by nearly 50 %. If, however, care is taken to determine the reaction, either electrometrically or by the use of an external indicator, the sulphuric acid neutralisation is satisfactory. To avoid this operation, precipitation by carbon dioxide after making alkaline with ammonia may be used (precipitation with carbon dioxide from the solution without addition of ammonia is incomplete). The filtrate from the final boiling with barium hydroxide, representing 10.7 kg. placenta, was divided into two equal portions. The first (No. 336 *a*) was freed from barium in the usual way by sulphuric acid (volume 110 cc., diluted 12 times for standardisation; 6 doses of 0.2 cc. to each of 19 rats; estimated unit 0.19 cc. approx.; solids 0.38 mg., 0.032 mg. *N* per cc.). To the second (No. 336 *b*) a dilute solution of ammonium hydroxide was added until the solution smelt definitely of ammonia. Carbon dioxide was then passed through the solution until precipitation of the barium was complete, when the precipitate of barium carbonate was filtered off and the ammonia quantitatively removed from the filtrate by distillation in a current of steam. The residual solution gave no precipitate with sulphuric acid. (Volume 80 cc.; for standardisation, diluted 18 times; 6 doses of 0.2 cc. each to 20 rats; estimated unit 0.1 cc. undiluted; solids 0.56 mg., 0.021 mg. *N* per cc.) This experiment shows that the latter method of removing barium is also satisfactory.

*The method finally adopted.* This is based on the experiments described above, and the procedure is described in detail, since the exact conditions are of importance, for a quantity of 10 kg. of dissected and minced placenta; this is added to 10 litres of hot water containing in solution 200 g. of crystalline barium hydroxide. The mixture is boiled for 2 hours in a steam-heated, enamelled iron vessel, furnished with a reflux condenser, and is afterwards poured on to filters made of "jean" (filter paper may be used for small quantities of material). The liquid filters readily, and the filtrate is concentrated in a shallow, steam-jacketed, enamelled iron pan to a volume of about 4 litres. This, together with the slight precipitate which may have separated, is extracted by mechanical

shaking for periods of  $\frac{1}{2}$  hour with three or four successive quantities, each of 1 litre, of butyl alcohol. The combined extracts are distilled under diminished pressure until nearly all the butyl alcohol is removed and the residue is dissolved in 2 litres of hot water and is filtered through a folded paper. The precipitate is washed with ether and the ethereal extract is added to that obtained from the aqueous portion (by shaking in a separating funnel with three or four successive quantities, each 0.5 litre, of ether). The combined ether extract is washed with water<sup>1</sup> (two washings of about 500 cc. each) and is then concentrated (transferring to a small flask at the end of the evaporation) to a slight, oily residue which is freed from the remaining trace of butyl alcohol by distilling *in vacuo* with a little water. The residue is dissolved in a minimum amount of alcohol (about 5 cc.) and is transferred to a volume of about 200 cc. water. The suspension thus obtained is shaken with three successive quantities of about 300 cc. ether, and the combined extracts are then washed three times with about 50 cc. of approximately 0.1 *N* hydrochloric acid, and finally with distilled water until the washings are free from chloride. The ethereal solution is then evaporated and the very small residue is dissolved in a minimum quantity of alcohol (2 or 3 cc.) and the alcoholic solution is poured into about 100 cc. of distilled water, and at this stage the extraction with ether may be repeated a third time if desired. This is not generally necessary, however, and after removing nearly all the alcohol by evaporation from an open dish on the water-bath, 10 cc. of a 5 % solution of crystalline barium hydroxide in water are added to the aqueous suspension, and the mixture is heated under reflux in a boiling water-bath or is gently boiled on a sand-bath for a period of 1 to 2 hours. The contents of the flask are then cooled to room temperature, and the clear solution is filtered from the slight precipitate which has formed. The filtrate is set on one side and the precipitate is thoroughly extracted with successive small quantities of hot alcohol. The alcoholic extract is evaporated to small volume and is then added to about 50 cc. of distilled water; 5 cc. of 5 % barium hydroxide are added and the mixture is boiled as before for 1 hour, when it is cooled and filtered. The combined filtrates from the first and second boilings with barium hydroxide are next heated on the boiling water-bath and the barium is removed by either of the methods described. The final product should be quite free from traces of barium or of sulphuric acid, and is usually faintly acid in reaction (about  $p_H$  5). The results obtained from 16 typical batches are given in Table V, Nos. 280 to 292.

*Physical properties.* The clear liquid obtained as described above is colourless, or very pale yellow in colour, often showing a strong blue fluorescence. It may be evaporated in an open dish on the steam-bath giving a residue which retains the activity of the original preparation, is freely soluble in absolute alcohol, but does not redissolve readily in a small volume of water. The final product appears to be in the form of a solution (true or colloidal) since it passes through filter paper

<sup>1</sup> It may with advantage be washed with hydrochloric acid and water at this stage also.

with little or no diminution of activity, and may be filtered through a Berkefeld candle without great loss. Thus 560 cc. of material prepared as above, containing 7.0 units per cc. were filtered through a previously moistened Berkefeld candle, which had been tested to retain bacteria. The total volume thus obtained was 558 cc., and this, standardised on 52 rats, was shown to contain 5.0 units per cc. The loss was, therefore, about 35 %. This test was performed with an impure product containing 0.08 mg. of solids per unit. Further evidence as to solubility is given by dialysis. Some of the material, after filtration through the Berkefeld filter (20 cc.; 5.0 units per cc.) was placed within a Schleicher and Schüll parchment diffusion thimble (tested after the experiment and found not to allow egg-albumin to pass through) so that, after inserting the manometer tube, a slight pressure was indicated; this did not diminish during the experiment. The thimble was allowed to stand for 24 hours in a beaker containing 75 cc. of distilled water. At the end of this time the thimble was removed and its contents (the change in volume being negligibly small) were diluted three times, after which 6 doses of 0.2 cc. were given to each of 19 rats, three of which showed an oestrus response (before dialysis in double this dilution a similar injection into 52 rats caused a 50 % response). The outside solution (75 cc.) was injected without dilution as 6 doses of 0.2 cc. each into 18 rats, when 6 showed a definite oestrus response. The material is thus dialysable with some loss of activity. This is in agreement with the findings of Laqueur *et al.* [1926, 3; 1927]. The preparation is stable to boiling, and we have used this method, as well as heating in the autoclave at 120° for 15 minutes, for sterilising the product. Tricresol may be added without obvious effect on the activity. After filtration through the Berkefeld candle, the material described above was divided into two portions to one of which 0.1 % of tricresol had been added, and both were sealed in sterile glass vessels and placed in an incubator at 37° for 3 months. At the end of this time they were removed and after diluting the contents six times with water, 6 doses of 0.2 cc. were given to each rat. The specimen without tricresol (injected into 40 rats) produced a 58 % response; that with the tricresol (36 rats) gave a 44 % response, whereas standardisation before incubation (52 rats) showed oestrus in 50 % of the animals. These variations, however, are within the limits of error of the method of standardisation.

*Ether extraction.* The active material is soluble in ether and may be completely removed from the aqueous solution of the hormone by continuous extraction with ether. The ethereal extract on evaporation yields a slight colourless residue which dissolves completely in warm water and the solution contains active material. This has not yet been fully investigated quantitatively; there appears, however, to be a considerable loss of activity, but this may possibly be accounted for by peroxides which were present in the samples of ether used.

*The nitrogen content.* As will be seen from the last two columns of Table V the product made in this way always contains nitrogen, as a rule to the extent of 5 or 10 % of the total solids. This is, of course, no indication that the pure

hormone contains nitrogen, indeed a contrary result has been observed by Laqueur *et al* [1925] and by Allen and Doisy [1927], and as our analytical figures show, there is no relationship between the nitrogen content and the activity. The nitrogenous constituents appear to be non-volatile in steam, non-basic, or, if basic, to give a hydrochloride and sulphate soluble in ether, and about 15 % only of this total nitrogen is liberated by distillation with concentrated sodium or potassium hydroxides, the remainder being apparently stable to alkali. With the purest products the Millon reaction is exceedingly faint, whether performed in the usual way or with the modification of Weiss. The biuret reaction is negative. All batches have given a faint colour with Nessler's solution, the depth being of the same order as that given by a solution of ammonium sulphate containing 0.001 mg. nitrogen per cc. There does not appear to be any quantitative relationship between this coloration and the activity of the product. The main part of the nitrogenous constituent is extractable with ether from the aqueous solution. Investigation of these points is being continued.

Experiments upon the further purification of this material are in progress. Fractional precipitation with alcohol and ether shows that the potency can be greatly increased.

It may be noted that active material has been obtained from the urine of pregnant women by butyl alcohol extraction, followed by the process described earlier. Two litres of urine, when so treated, gave 240 cc. of aqueous solution of which 6 doses of 0.2 cc. given to 20 rats produced 12 oestrus responses. This corresponds to 120 units per litre, and hence confirms the work of Zondek [1928].

The preparation has been used clinically in several centres. Whilst it is as yet too early to state the results, experiments have proved that large doses may be given daily to women without any untoward results. The clinical reports up to date indicate that the preparation will have a definite application in the treatment of various disorders such as amenorrhoea and premature menopause. This aspect will be dealt with elsewhere.

#### SUMMARY.

1. A method is described whereby the oestrus-producing hormone is constantly obtainable in good yield from placenta in the form of an aqueous solution.

2. This material is inactive in a single dose but produces oestrus when given as a series of small injections. Standardisation of this product is discussed and the use of a method combining the multiple dose technique with the statistical method is described.

3. The weight of one unit as calculated on the basis of this method of standardisation is reduced to 0.01 mg.

4. The physical and chemical properties of this preparation are described, and it is shown that active material will pass through a porcelain filter and that it may be dialysed through parchment.

5. The material is stable to prolonged incubation and to short exposures to high temperatures.

6. The substance is without effect on blood-pressure or respiration and no ill effects have been observed when it has been injected daily for some months into patients.

We have to thank the following hospitals for allowing us to collect material from them: City of London Maternity Hospital; East End Maternity Hospital, Commercial Road; General Lying-in Hospital, York Road; Queen Charlotte's Maternity Hospital; Queen Mary's Hospital for the East End, Stratford.

It is a great pleasure to acknowledge the help afforded to us by Messrs Boots Pure Drug Company, Nottingham. One of us (F. O. H.) held the Boots Scholarship in these laboratories and the Company gave us the technical assistance of Mr E. A. Harvey. In addition, they assisted very generously with the heavy laboratory expenses in connection with this work.

#### ADDENDUM.

When this paper was completed, the communication of Thayer, Jordan and Doisy [1928] came to our notice. These authors convert their very active, oily material into water-soluble form, by a method which presents many similarities to that used by Laqueur and his co-workers [1927]. The experiments described above have shown that our material, the unit of which is 0.01 mg. or more, is inactive when given in one dose or by the method described by Allen *et al.* [1924]. Unfortunately Thayer, Jordan and Doisy give no details of standardisation in the paper referred to and, therefore, it is not possible for us to compare the potency of their material with that of our own.

#### REFERENCES.

- Allen, Byron, Robertson, Colgate, Johnston, Doisy, Kountz and Gibson (1924). *Amer. J. Anat.* **34**, 133.  
Allen and Doisy (1927). *Physiol. Rev.* **4**, 600.  
Coward and Burn (1927). *J. Physiol.* **63**, 270.  
Dickens, Dodds and Brinkworth (1927). *Lancet*, i, 1015, 1182.  
Dickens, Dodds and Wright (1925). *Biochem. J.* **19**, 853.  
Dodds (1928). *Lancet*, i, 1107.  
Frank, Gustavson, Holloway, Hyndman, Krueger and White (1926). *Endocrinology*, **10**, 260.  
Glimm and Wadehn (1926). *Biochem. Z.* **179**, 3.  
Laqueur, Hart, de Jongh and Wijsenbeck (1925). *Proc. K. Akad. Wetensch. Amsterdam*, **28**, 890.  
——— (1926 1, 2, 3). *Deutsch. med. Woch.* **52**, 4, 52, 1331.  
Laqueur, Hart and de Jongh (1927). *Lancet*, i, 1126.  
Loewe (1925). *Zentr. Gynäk.* **49**, 1735.  
Parkes (1928). *Biol. Rev.* **3**, 208.  
Ralls, Jordan and Doisy (1926). *J. Biol. Chem.* **69**, 357.  
Thayer, Jordan and Doisy (1928). *J. Biol. Chem.* **79**, 53.  
Zondek (1928). *Klin. Woch.* **7**, 1404.  
Zondek and Brahn (1925). *Klin. Woch.* **4**, 2445.

# CXCI. VARIATIONS IN SOME OF THE CONSTITUENTS OF THE BLOOD THROUGHOUT THE MENSTRUAL CYCLE IN NORMAL WOMEN.

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*(Received November 5th, 1928.)*

DURING the course of an investigation on certain forms of Bright's disease, it became necessary to know the possible variations in some of the constituents of the blood which might occur throughout the menstrual cycle. As figures on the subject were not readily available, it was decided to undertake the necessary investigations in a group of normal women. The results obtained are considered of sufficient general interest to justify publication as they may prove of value to other workers. A further point of interest has arisen out of the investigation as corresponding determinations were made in a series of women of a different social class who, though not suffering from any recognisable disease, were nevertheless in a state of subnormal health due largely to overwork, worry, insufficient food, and living generally under adverse circumstances. The figures obtained in the two groups differ in some important respects. Those for the latter group will be published in due course.

Determinations of the following constituents of the blood-serum were made—bicarbonate, chloride, urea, calcium. In a few instances the inorganic phosphorus of the serum was also estimated. The subjects selected were all young, healthy, athletic women, thirteen in number, pupils of the Massage Training School at Guy's Hospital, who very kindly volunteered to undergo investigation. None of them had suffered at any time from menstrual irregularities or abnormalities, or had recently had any illness which might have been expected to disturb the menstrual cycle. No dietetic restrictions were imposed. The blood was usually collected at midday, under paraffin, from a vein in the antecubital fossa, without exerting pressure, and the determinations were always made within a few hours of taking the blood. Observations were usually made midway between menstrual periods, or on the 1st, 2nd, or 3rd day of the period. In each case the observations covered at least two complete menstrual cycles.

## METHODS.

*Serum-bicarbonate.* The titration method of Van Slyke, Stillman and Cullen [1919] was used throughout. The titration was taken to a constant end-point,  $p_H$  7.4, instead of to the  $p_H$  of the sample of blood used. To a slight

extent this would render the figures in "acid" samples too high, and in "alkaline" samples too low. In practice however the error is not great, and would tend to minimise the findings, so that a decrease in the bicarbonate would be less marked, using this modification, than that actually present. Phenol red was used as an indicator instead of neutral red. The results are expressed as molar concentration of bicarbonate.

*Serum-chloride.* Claudius's method [1924] was used.

*Inorganic phosphate.* Briggs's modification of the Bell and Doisy method [1920] was used.

*Serum-calcium.* Kramer and Tisdall's method [1921] was used.

*Urea.* Twort and Archer's method [1923] was used.

Our own normals agree with those of other workers using these several methods, but, as will be seen, there is reason to believe that in women of the child-bearing age a wider range of normal variation is met in some of the blood-constituents, notably the bicarbonate and chlorides, as compared with the corresponding variations in males. It has been shown previously [Cook and Osman, 1923], and is confirmed here, that the average normal bicarbonate in women is slightly lower, and is subject to greater variation than in the male.

## RESULTS.

As will be seen from the accompanying tables, on the whole there is very little change in these constituents of the blood during the menstrual flow in perfectly healthy women. There appears to be no significant change in the blood-calcium and blood-urea. There is a slight but definite increase in the serum-chlorides during the flow, and to some extent a slight decrease in the serum-bicarbonate. There would also appear to be no significant difference in the figures obtained on the 1st, 2nd, or 3rd days of the menstrual flow. No change was observed in those cases in which the inorganic phosphate of the serum was estimated. The results are summarised in the two following tables.

## SUMMARY.

In healthy adult females there is a slight decrease in the serum-bicarbonate, and a corresponding slight increase in the serum-chloride during the menstrual flow. The serum-calcium and serum-urea show no significant change at this time.

We wish here to record our indebtedness to those ladies who so kindly volunteered to have the investigations done at no little personal inconvenience.

This investigation was carried out with the aid of a grant from the Medical Research Council.



Table I. *Serum-bicarbonate, -chloride, -calcium and -urea in normal women throughout the menstrual cycle.*

			NaHCO <sub>3</sub> M	NaCl g. per 100 cc.	Urea mg. per 100 cc.	Ca mg. per 100 cc.
E. M.	Period	1	0.0277	0.547	26	11.9
		2	0.0296	0.600	28	10.1
	Midway	1	0.0300	0.577	36	10.6
		2	0.0301	0.576	29	10.8
F. M.	Period	1	0.0287	0.599	30	10.7
		2	0.0294	0.565	31	10.4
	Midway	1	0.0306	0.541	34	—
		2	0.0302	0.614	32	10.4
M. B.	Period	1	0.0298	0.573	35	10.4
	Midway	1	0.0299	0.573	28	—
E. W.	Period	1	0.0278	0.582	30	—
		2	0.0290	0.585	28	10.5
	Midway	1	0.0301	0.576	32	9.9
		2	0.0303	0.582	32	10.4
N. K.	Period	1	0.0292	0.611	36	10.3
		2	0.0302	0.614	35	10.3
	Midway	1	0.0300	0.600	42	8.6
		2	0.0296	0.598	32	11.3
G. M.	Period	1	0.0260	0.597	33	—
		2	0.0276	0.579	26	11.2
	Midway	1	0.0290	0.570	35	10.3
		2	0.0310	0.611	35	10.7
D. M.	Period	1	0.0308	0.618	42	10.6
	Midway	1	0.0280	0.556	25	9.8
		2	0.0308	0.594	26	9.4
		3	0.0296	0.614	32	9.9
F. P.	Period	1	0.0300	0.608	34	10.5
		2	0.0295	0.614	33	10.6
	Midway	1	0.0290	0.611	35	11.3
		2	0.0317	0.579	31	11.6
G. D.	Period	1	0.0297	0.603	26	10.2
		2	0.0283	0.632	27	10.3
	Midway	1	0.0289	0.605	29	9.9
		2	0.0310	0.614	30	10.3
M. G.	Period	1	0.0283	0.620	45	10.0
		2	0.0296	0.605	49	9.7
	Midway	1	0.0298	0.617	53	10.6
		2	0.0304	0.591	48	10.5
E. H.	Period	1	0.0281	0.623	32	10.6
		2	0.0292	0.589	34	10.3
	Midway	1	0.0312	0.570	30	10.5
		2	0.0302	0.597	34	10.7
B. W.	Period	1	0.0294	0.567	29	9.7
		2	0.0321	0.579	28	9.6
	Midway	1	0.0317	0.620	26	10.0
		2	0.0305	0.565	28	10.6
E. R.	Period	1	0.0283	0.611	32	10.4
		2	0.0290	0.608	31	10.9
	Midway	1	0.0281	0.576	33	10.5
		2	0.0284	0.562	32	10.6

N.B. The two figures in each column refer to readings taken at two separate periods or intermenstrual periods. Each individual figure is the average of at least two, generally of three, readings.

Table II. *Average values for serum-bicarbonate, -chloride and -calcium. Menstrual and intermenstrual.*

	Intermenstrual			Menstrual		
	Highest	Lowest	Average	Highest	Lowest	Average
M NaHCO <sub>3</sub>	0.0317	0.0280	0.0301	0.0321	0.0260	0.0291
NaCl (g. per 100 cc.)	0.620	0.541	0.585	0.623	0.547	0.591
Calcium (mg. per 100 cc.)	11.6	8.6	10.4	11.9	9.6	10.4

## REFERENCES.

- Bell and Doisy (1920). *J. Biol. Chem.* **44**, 55.  
 Claudius (1924). *Acta Med. Scand.* **61**, 3.  
 Cook and Osman (1923). *Guy's Hosp. Rep.* **73**, 233.  
 Kramer and Tisdall (1921). *J. Biol. Chem.* **47**, 475.  
 Twort and Archer (1923). *Lancet*, i, 1102.  
 Van Slyke, Stillman and Cullen (1919). *J. Biol. Chem.* **38**, 167.

# CXCII. CHANGES IN THE NUCLEAR-PLASMIC RATIO OF VERTEBRATE POIKILOTHERMS DURING HUNGER.

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## INTRODUCTION.

THE metabolic processes of poikilotherms differ considerably from those of homeotherms. Whilst during hunger proteins, carbohydrates and fats are metabolised by warm-blooded animals, yet the relative consumption of these constituents changes greatly during the course of inanition. Thus glycogen and fats are metabolised during the first or middle stage of inanition, and, as these reserves are consumed, protein metabolism begins to predominate. In the case of poikilotherms, conditions are different. The work of Reuss and Weinland [1912], Knauthe [1898], Białaszewicz [1919] and others, has shown that the part played by protein in hunger metabolism is several times greater than for homeotherms, and recently Przylecki and Karczewski [1923] and Przylecki and Opienska [1926] have shown that, only under certain abnormal conditions, vertebrate poikilotherms are able, using fat and carbohydrate reserves, to spare the utilisation of protein in hunger as a source of energy.

A higher percentage of the organic constituents of poikilotherms is represented by protein than is the case for homeotherms. This is illustrated by Table I, where percentage protein and percentage protein metabolism in hunger are compared for various animals [Białaszewicz, 1919].

Table I. *Protein content and percentage protein metabolism.*

Animal	% of protein in organic constituents	% of total hunger metabolism due to protein	Author
<i>Lepus caniculus</i>	71.8	20.5*	Rubner [1881]
<i>Gallus domesticus</i>	66.8	18.8*	Kückein [1882]
<i>Anguilla vulgaris</i>	84.6	83.1	Reuss and Weinland [1912]
<i>Salmo salar</i>	63.3	73.1	Miescher [1897]
<i>Hirudo medicinalis</i>	90.5	84.5	Białaszewicz [1919]
<i>Amblystoma</i>	88.6	89.1	Librach [1922]
<i>Rana esculenta</i>	86.2	85.5	Librach [1922]

\* For first few days of hunger.

We see that protein represents 80-90 % of the organic constituents of poikilotherms, and that about the same percentage of the total hunger

metabolism is due to protein. The loss of weight on death from inanition is much greater than for homeotherms, being 55–75 % of the original weight for frogs, axolotls and newts [Librach, 1922], whilst for birds and mammals it does not exceed 40–50 %.

The question arises whether the difference in the loss of proteins accompanying inanition between homeotherms (about 40 %) and poikilotherms (60–70 %) is due to a larger protein reserve possessed by the latter class of animals. Should this be so, the exhaustion of this reserve should lead to a marked increase in the nuclear-plasmic ratio. Research on this question has been conducted in this laboratory for some time, along lines initiated by Schaeffer and Le Breton [1923]. Truszkowski [1928] found that the nuclear-plasmic ratio of frogs not only did not rise, but even fell at the end of winter, from the average value of  $37.8 \times 10^{-3}$  to  $35 \times 10^{-3}$  in March and  $30.5 \times 10^{-3}$  in April. This paper describes further similar researches on protein reserve, the number of different species examined being greater, and the period of inanition longer. A wider range of temperatures of the surroundings was also taken.

#### EXPERIMENTAL.

Frogs (*Rana esculenta*), axolotls (*Amblystoma*) and trout (*Salmo officinalis*) were taken as experimental material. The technique adopted was that described by Truszkowski [1926], being a slight modification of Schaeffer and Le Breton's method.

1. *Frogs*. Four series of experiments were carried out on frogs. The first group represented fed animals, taken in October and November, the second group was of frogs not fed for 26 days, kept at a temperature of 25° (December), the third represented 53 days of hunger at 15° (January), and the fourth 73 days of hunger at 0° (February). A few animals were taken for each determination, being minced immediately after removal of the testes or ovaries, and of the contents of the alimentary canal. The results, given in Table II, show that, as Truszkowski found, no great variations occur in the nuclear-plasmic ratio, the values found for the above four groups being respectively 35.89, 36.07, 33.37 and  $36.77 \times 10^{-3}$ .

Table II. *Nuclear-plasmic ratio of frogs at various stages of inanition.*

No.	Days hunger	% solid substance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear- plasmic ratio	Remarks
1	0	21.80	2.633	12.08	98.76	453	$39.0 \times 10^{-3}$	
2	0	21.15	2.954	13.97	90.30	427	$31.52 \times 10^{-3}$	
3	0	21.30	2.758	12.95	98.85	464	$37.17 \times 10^{-3}$	
Mean	0	21.42	2.782	13.0	95.97	448	$35.89 \times 10^{-3}$	
4	26	20.20	2.573	12.49	88.21	437	$36.07 \times 10^{-3}$	At 25°
5	53	20.23	2.651	13.14	85.24	425	$33.37 \times 10^{-3}$	At 15°
6	73	22.10	2.627	11.83	92.98	420	$36.77 \times 10^{-3}$	At 0°
Mean	—	20.84	2.617	12.49	88.81	427	$35.4 \times 10^{-3}$	

The figures given by Scaffidi [1910] for the purine-nitrogen content of frog muscle (35 mg. %) are too low, this difference being due to the fact that he calculated  $N_p$  as the sum of that of the extracted muscles plus that of the unhydrolysed extract.

2. *Axolotls*. A considerably longer period of inanition was taken for axolotls. As for frogs, four series of determinations were made. The first group of animals was analysed before hunger; the remainder was divided into three groups, each member of which was placed in a separate vessel with water at room temperature, which was changed every few days. These groups were taken for analysis after 145, 280 and 340 days of hunger. Our observations upon axolotls are to a certain extent divergent from those of Librach, who found that no individual survived more than 173 days at 25°, when 75 % of the original weight had been lost; in our case, after 340 days of hunger only 56.6 % had been lost. Further, Librach did not find a diminution in total dry content, which in our case fell from 13.5 to 9.1 %.

The results, given in Table III, show that the nuclear-plasmic ratio rises from 33.0 to  $38.0 \times 10^{-3}$  after the first 5 months of hunger, and then falls to  $28.5 \times 10^{-3}$  after 11 months. Owing to an accident, values were not obtained for 9 months of hunger, and also unfortunately material was not available for the study of the final stages of inanition, which would have been particularly interesting in view of the pre-mortal rise in nitrogen elimination.

Table III. *Nuclear-plasmic ratio of axolotls at various stages of inanition.*

No.	Days hunger	% loss in weight	% solid substance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear-plasmic ratio
1	0	—	13.80	1.702	12.33	52.38	380	$31.75 \times 10^{-3}$
2	0	—	13.83	1.679	12.07	56.66	410	$34.93 \times 10^{-3}$
3	0	—	13.60	1.774	13.06	55.57	408	$32.32 \times 10^{-3}$
Mean	0	—	13.74	1.718	12.49	54.87	400	$33.0 \times 10^{-3}$
4	145	35.5	13.50	1.732	12.83	63.48	471	$38.0 \times 10^{-3}$
5	280	55.2	10.20	1.282	12.57	—	—	—
6	340	56.6	9.09	1.197	13.17	33.27	366	$28.5 \times 10^{-3}$

3. *Trout*. Trout were obtained from the Zloty Potok breeding ponds. The fish, which were in a good state of nutrition, consisted of small fry about 5 g. weight each, one-year-olds, weighing 100–150 g., and two-year-olds weighing about 300 g. These were kept in running water aquaria which the Ichthyological Laboratory of the Chief Agricultural School kindly placed at our disposal. The first group of trout were analysed at once (Nov. 25th to Dec. 13th); the remaining fish were subjected to inanition.

50–100 g. of fresh mince were taken for each determination. The roe was removed before mincing, as well as the contents of the alimentary canal. The results, given in Table IV, indicate a 36.3 % increase in the nuclear-plasmic ratio of the young fish, which rises from  $38.6 \times 10^{-3}$  for fed fish to  $52.5 \times 10^{-3}$  after 80 days of inanition, involving a loss of body weight of 30 %. In one case, where the fish were analysed several hours after death, the nuclear-

Table IV. *The nuclear-plasmic ratio of trout fry at various stages of inanition.*

No.	Days hunger	% loss in weight	% solid sub- stance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear- plasmic ratio	Remarks
1	—	—	21.0	2.78	13.24	105.18	501	$39.32 \times 10^{-3}$	
2	—	—	—	2.58	—	97.05	—	$39.34 \times 10^{-3}$	
3	—	—	21.67	2.64	12.19	113.5	524	$44.91 \times 10^{-3}$	
4	—	—	21.53	2.57	11.95	86.19	401	$34.70 \times 10^{-3}$	
5	—	—	21.8	2.72	12.48	93.05	430	$35.66 \times 10^{-3}$	
Mean	—	—	21.49	2.66	12.46	99.23	464	$38.78 \times 10^{-3}$	
6	80	30	18.3	2.46	13.31	123.12	673	$52.49 \times 10^{-3}$	
7	80	30	17.65	2.30	13.03	97.4	551	$44.21 \times 10^{-3}$	Dead fish taken

Table V. *The nuclear-plasmic ratio of two-year-old trout at various stages of inanition.*

No.	Days hunger	% solid sub- stance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear- plasmic ratio	Remarks
1 ♀	—	22.15	2.79	12.6	88.0	397	$32.2 \times 10^{-3}$	
2 ♀	—	19.4	2.42	12.38	53.11	274	$22.44 \times 10^{-3}$	Dead fish taken
3 ♀	—	21.7	2.83	13.04	74.0	341	$26.82 \times 10^{-3}$	Dead fish taken
4 ♀	80	22.0	2.82	12.83	121.5	552	$45.0 \times 10^{-3}$	

Table VI. *The nuclear-plasmic ratio of yearling trout at various stages of inanition.*

No.	Days hunger	Days subse- quent feeding	% solid substance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear- plasmic ratio
1	90	—	17.7	2.525	14.27	113.5	641	$47.06 \times 10^{-3}$
2	180	—	17.0	2.61	15.36	88.5	520	$35.09 \times 10^{-3}$
3	30	60	19.7	2.58	13.1	116.5	592	$47.15 \times 10^{-3}$
4	30	150	20.17	2.92	14.49	88.72	440	$31.3 \times 10^{-3}$

plasmic ratio was only  $44.2 \times 10^{-3}$ , indicating rapid *post mortem* purinolysis or deamination. Similar results were obtained for two-year-old fish (Table V). Thus, before inanition, a nuclear-plasmic ratio of  $32.2 \times 10^{-3}$  was found for a female trout, whereas after 80 days of hunger this value had increased by 40.1 % to  $45.0 \times 10^{-3}$ . Here also fish taken for analysis some hours after death had very low purine contents. After 90 days of inanition the nuclear-plasmic ratio of one-year-old fish (Table VI) was  $47.06 \times 10^{-3}$ ; after 180 days when the fish were in the last stages of exhaustion, this value falls again to  $35.09 \times 10^{-3}$ . After 30 days' hunger followed by 60 days' feeding with liver, the nuclear-plasmic ratio was  $47.15 \times 10^{-3}$ , whilst after 150 days of restitution it was  $31.3 \times 10^{-3}$ . Owing to lack of material, separate determinations of the nuclear-plasmic ratio of one-year-old fish were not made; these presumably differ little from the values obtained for fry and for two-year-olds, or from those yearlings subjected to prolonged restitution. There is thus an undoubted increase in the value of the nuclear-plasmic ratio during the first stages of hunger, followed by a diminution to the normal value or slightly below. Restitution takes place slowly, pointing to an early depletion of reserves.

## DISCUSSION.

A consideration of the above results, both from the points of view of protein reserve and of the part played by nuclear and plasmic constituents in hunger metabolism, shows that the animals studied may be divided into two groups—amphibia, which exhibit a practically constant nuclear-plasmic ratio throughout the duration of inanition, and fish, whose nuclear-plasmic ratio undergoes marked variations.

Certain differences could, however, be distinguished within the former group. Thus the nuclear-plasmic ratio of frogs was practically constant over the period of hunger, whilst in the case of axolotls, it rose by 15 % after 5 months, to fall again to 15 % below normal after 11 months of hunger. This decline may be observed for all animals so far studied, trout also exhibiting it, and Truszkowski, who found the same for rats and frogs, explains it as being due to the disintegration of a certain number of cells, with elimination of nuclear constituents and resorption of plasmic constituents by other cells; this process would prevent the excessive increase of the nuclear-plasmic ratio. It is also, however, possible that this fall is due to elimination of extra-nuclear purines. The constancy of this ratio for the frog would indicate that this animal has a more economical hunger metabolism than the axolotl, and is able, by a more uniform oxidation of its constituents to achieve a better regulation of its hunger metabolism. This view is further supported by Librach's observation that a tendency exists in these animals towards the reduction of their nitrogen metabolism during the last stages of hunger; it may be, however, that the protein reserve of frogs is very small, if it exists at all.

On the whole, our results for amphibia diverge considerably from those obtained by other authors, who, on the basis of measurements of the surface or volume of nucleus and cytoplasm, found that hunger in all cases led to an increase in the nuclear-plasmic ratio [Vieweger, 1921 (infusoria); Morgulis, 1911 and Leonard, 1887 (amphibia)]. Our gravimetric determinations would rather support the work of Białasiewicz and of Librach, who found that the chemical composition of fed and starved organisms is practically identical, and who connected this fact with the ability to withstand protracted inanition.

The results for trout are quite different. All individuals, irrespective of age, exhibited a considerable though transient increase of nuclear-plasmic ratio, amounting to about 40 %, followed by a return to normal values. So considerable a change in a fish closely related to the salmon, which has served as the classical example of the existence of reserve protein, must, in our opinion, be considered as a further, definitive proof of the existence of this reserve protein.

Both the chemical and histological evidence adduced in support of the reserve protein theory by Miescher [1897], Paton [1898], C. W. Greene [1919] and C. H. Greene [1919], have been considered to constitute a sufficient basis for the acceptance of this theory. In our opinion, however, certain objections might be raised against the validity of these proofs.

Firstly, the fact that the solid content of tissues falls continuously during hunger to a large extent vitiates conclusions based upon morphological evidence, as Schaeffer and Le Breton have pointed out. For the same reason, the observation that 30 % of the relative protein content is lost, taking as the basis for comparison the whole of the remaining fat-free protein (C. W. Greene's "protoplasm basis") cannot be accepted as evidence for the existence of reserve protein. C. W. Greene considers the constant mineral content of starved animals to be a sufficient proof that no change has occurred in the degree of hydration of the cytoplasmic protein colloids, but it may be considered that even minimal changes in the relative contents of the particular mineral constituents may lead to an increase in the water-content of the tissues.

The results of our research, based upon the determination of changes in the ratio of the weights of substances independent of the degree of hydration of the tissues, support the hypothesis of the existence of reserve protein, at least in fish, and their agreement with the results of other authors shows that the method applied by us to the study of this question is, in spite of all its short-comings, suitable for the purpose in question. These defects will be discussed elsewhere, and deal chiefly with extra-nuclear purines.

#### SUMMARY.

1. In view of their higher protein content, and of the greater part played by protein in the hunger metabolism of poikilotherms than in that of homeotherms, as well as of the greater loss of weight attainable, it would appear that the former afford a convenient material for the study of protein reserve.

2. The effect of inanition upon the nuclear-plasmic ratio of amphibia (*Rana esculenta* and *Amblystoma*) and of fish (*Salmo officinalis*) was investigated.

3. The nuclear-plasmic ratio was practically constant for frogs; in the case of axolotls it rose by 15 % after 5 months of hunger, and fell to 15 % below normal after 11 months of hunger.

4. The nuclear-plasmic ratio of trout after 80 days of hunger rose by 30 % for fry and 49 % for two-year-olds. Yearling trout exhibited an increase of about 50 % after 90 days of hunger. This rise in the nuclear-plasmic ratio is transient, values obtained for the final stages of hunger being subnormal.

5. A short period of restitution does not reduce the nuclear-plasmic ratio of trout to its normal value; this, however, takes place after a prolonged period of restitution.

6. The results obtained for trout are, in view of the elimination of the effect of tissue dilution accompanying hunger, satisfactory proof of the existence of reserve protein.

I wish to express my sincere gratitude to Prof. S. J. Przylecki at whose suggestion this research was undertaken, and to whose unfailing advice and help I am indebted for its conclusion.



## REFERENCES.

- Białasiewicz (1919). *Trav. Soc. Sci. Varsovie*, 3, No. 32.  
Greene, C. H. (1919). *J. Biol. Chem.* 39, 457.  
Greene, C. W. (1919). *J. Biol. Chem.* 39, 435.  
Knauths (1898). *Arch. ges. Physiol.* 73, 490.  
Kückein (1882). *Z. Biol.* 18, 23.  
Leonard (1887). *Arch. Anat. Physiol. Suppl.* Vol. 28.  
Librach (1922). *Trav. Inst. Nencki, Varsovie*, 1, No. 13, 4.  
Miescher (1897). *Histolog. Arb.* 2, 116.  
Morgulis (1911). *Arch. Entw.-Mech. Org.* 32, 169.  
Paton (1898). *Rep. Fishery Board, Scotland*, 139.  
Przylecki and Karczewski (1923). *Arch. Internat. Physiol.* 22, 208.  
Przylecki and Opienska (1926). *Bull. Acad. Sci. Pol. B*, 293.  
Reuss and Weinland (1912). *Z. Biol.* 59, 283.  
Rubner (1881). *Z. Biol.* 17, 214.  
Scaffidi (1910). *Biochem. Z.* 30, 473.  
Schaeffer and Le Breton (1923). *Trav. Inst. Physiol. Strasbourg. Variations biochimiques du rapport nucléo-plasmatique.*  
Truszkowski (1926). *Biochem. J.* 20, 437.  
—— (1928). *Biochem. J.* 22, 198.  
Vieweger (1921). *Trav. Inst. Nencki, Varsovie*, 1, No. 9, 1.

## CXCIH. CHANGES IN THE NUCLEAR-PLASMIC RATIO OF MAMMALS DURING HUNGER.

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IN the preceding paper [1928] upon changes in the nuclear-plasmic ratio of vertebrate poikilotherms during hunger considerable differences were observed in the behaviour of frogs, axolotls and trout. Thus the nuclear-plasmic ratio of amphibia remained practically constant during inanition, whilst in the case of trout it at first rose, falling to the normal value in the later stages of hunger. This result was interpreted as being evidence for the possession by trout of protein reserves.

In view of the smaller part played by protein in the metabolism of mammals, it appeared probable that the corresponding changes in their nuclear-plasmic ratio would be inconsiderable. Truszkowski's researches [1926, 1927], in which the nuclear-plasmic ratios of whole rats subjected to total inanition and of skeletal muscle and liver of dogs, fed on carbohydrates or proteins, or starved, were determined, appear to confirm this supposition. This author, who found that in skeletal muscle the nuclear-plasmic ratio remained constant under the above conditions, drew the conclusion that protein reserves do not occur in these tissues; the possibility of a limited storage of protein in canine liver was not, however, excluded. These results, are, on the whole, in accordance with generally accepted views [cf. Caspari and Stilling, 1925].

Deuel *et al.* [1928] found, on the other hand, that injections of thyroxine have the effect of increasing urea-nitrogen excretion without raising uric acid-nitrogen excretion, and this effect may be obtained even on persons previously subjected to prolonged nitrogen hunger. These results are, in spite of certain objections, such as that purine-nitrogen was not determined in the urine and that the residual nitrogen content exhibits a considerable increase after injection of thyroxine, strong evidence for the presence of protein reserves in man, and calculation shows that these must be to a considerable extent localised in the muscles.

Certain of Truszkowski's results necessitated additional research, chiefly as to the study of the nuclear-plasmic ratio during the various stages of hunger, and not only at death from inanition, and this question is, amongst others, the subject of this paper.

It is known that the part played by protein in hunger metabolism depends to a large extent upon the stage of inanition, and that it increases rapidly as fat reserves approach exhaustion; this is, according to Voit [1901], the cause of the premortal increase in nitrogen excretion, although Schultz [1899, 1901] and Serio [1923] ascribe the latter to the toxic effect of nitrogen hunger, which leads to the wholesale breakdown of cells.

The nuclear-plasmic ratio might increase to a varying extent, according to the slower or faster exhaustion of protein reserves, and might finally, during the pre-mortal period of widespread cell destruction, possess its normal value, or even a lower value (Truszkowski).

In order to throw light upon this matter the nitrogen excretion of fasting animals was determined parallel to their nuclear-plasmic ratio.

The chemical nuclear-plasmic ratio, expressed as  $N_P/(N_T - N_P)$ , does not, as Schaeffer and Le Breton [1923] have pointed out, express the true ratio of nuclear to cytoplasmic nitrogen. Accepting quite arbitrarily for technical reasons that purine-nitrogen is a measure of nuclear mass (in reality it amounts to only two-thirds of the total nucleic acid-nitrogen, the remainder being pyrimidine base-nitrogen), and that total nitrogen is expressive of the cytoplasmic mass, we obtain figures divested of any intrinsic significance. Only changes in the ratio can be considered as having any significance in the investigation of such questions as that of the existence of reserve protein. In order, however, for the figure obtained for purine-nitrogen to be as representative as possible of the nucleus, it should be derived from the nuclein fraction only, and would consist of guanine and adenine only; oxypurines such as hypoxanthine, xanthine and uric acid should be excluded, as well as the adenine of adenylic acid [Embden and Zimmerman, 1927, 1, 2].

Schaeffer and Le Breton, who worked on embryos, could neglect this second fraction, but point out that for adult animals it may be considerable, and this would be the more so for determinations of the chemical nuclear-plasmic ratio of skeletal muscle.

Experiments were therefore carried out in order to verify whether the results obtained for skeletal muscle, after removal of soluble purines and non-protein nitrogenous substances, such as carnosine, carnitine, creatine, etc., are similar to those obtained for unextracted muscle.

#### EXPERIMENTAL.

Well nourished rabbits were taken as experimental material. They were kept in small cages at room temperature, under conditions of absolute inanition, *i.e.* without food or water, and were weighed daily. The urine was collected quantitatively and nitrogen determined every few days; towards the latter stages of hunger determinations were as far as possible made every day. Seven rabbits were killed after periods of hunger varying from 7 to 14 days, loss of weight of 17.8 to 28.4 %, four after 10–18 days, 40 % loss of weight. Of these, one was taken for analysis 1–2 hours after death from inanition, and

three in the last stages of exhaustion—these exhibited a distinct pre-mortal rise in nitrogen excretion. The animals were killed by injection of chloroform into the heart, the skin being then immediately removed and skeletal muscle taken from the limbs, as far as possible free from tendons and fat. The material was then passed through a mincing machine, and 50–100 g. were weighed out and placed in boiling 4.5 % sulphuric acid.

In certain cases the carcasses were first chilled to 0°, and the above operations were repeated at a low temperature. This technique did not, however, affect the values obtained for purine-nitrogen.

The livers were taken as a whole, after the removal of the gall-bladder. Total and purine-nitrogen contents were determined as before, using for the latter determination a modification of Krüger and Schittenhelm's method.

Where water-extraction was applied the procedure adopted was as follows. The comminuted tissue was thrown into 500 cc. of boiling water, and the whole filtered after 10–15 minutes' boiling. This operation was twice repeated using 100–200 cc. of water, the united filtrates were freed from protein by boiling after feeble acidification with acetic acid, and the residue of coagulated protein was added to the original residue, which was then treated as before for total and purine-nitrogen estimation.

Table I. *Rabbit skeletal muscle.*

No.	% solid substance	% N live weight	% N dry weight	Purine-N mg. per 100 g. live weight	Purine-N mg. per 100 g. dry weight	Nuclear-plasmic ratio	Initial weight g.	Final weight g.	% loss	Days hunger
I	24.7	3.826	15.43	110.5	447	$29.73 \times 10^{-3}$				
II	24.4	3.755	15.39	114.57	470	$31.19 \times 10^{-3}$				
III	24.4	3.830	15.70	119.1	488	$32.10 \times 10^{-3}$				
IV	25.3	3.740	14.78	109.75	434	$30.20 \times 10^{-3}$				
V	25.3	3.720	14.43	87.35	340	$24.01 \times 10^{-3}$				
Va	23.3	—	—	85.8	368	—				
Mean	24.63	3.774	15.13	104.5	424	$28.05 \times 10^{-3}$				
VI	24.8	3.89	15.65	136.7	551	$36.42 \times 10^{-3}$	1910	1570	17.8	10
♀ VII	27.7	3.555	13.8	110.9	400	$29.31 \times 10^{-3}$	2890	2050	28.4	14
♀ VIII	23.75	3.605	15.18	123.3	520	$35.4 \times 10^{-3}$	2140	1670	22.0	9
♂ IX	25.0	3.90	15.36	95.5	383	$25.1 \times 10^{-3}$	3030	2300	24.0	9
X	22.5	3.65	16.22	82.42	366	$23.08 \times 10^{-3}$	2450	1820	23.4	11
XI	27.8	4.04	14.53	91.97	331	$23.30 \times 10^{-3}$	4250	3450	18.8	7
Mean	25.26	3.773	15.12	106.8	438	$28.77 \times 10^{-3}$				
♀ XII	—	3.71	—	103.0	—	$28.7 \times 10^{-3}$	2760	1630	40.9	10
♀ XIII	28.35	3.84	13.5	100.16	350	$26.0 \times 10^{-3}$	2100	1230	41.4	18
♂ XIV	21.2	3.936	18.5	100.0	472	$25.71 \times 10^{-3}$	2620	1550	40.8	18
XV	26.3	3.776	14.32	103.0	392	$28.02 \times 10^{-3}$	2850	1700	40.3	18
Mean	25.28	3.815	15.44	101.5	401	$27.34 \times 10^{-3}$				

The results, given in Table I, are in almost every case the mean of 2–3 concordant determinations. Considerable individual differences in purine-nitrogen content exist, this varying from 85.8 to 119.1 mg. per 100 g. The mean value of 104.5 mg. is higher than that found by Kikuchi [1923], viz. 66 mg. with variations of 58–83 mg.; this author, however, used Burian and

Hall's method, which, according to Schaeffer and Le Breton [1923], gives low results. Rabbit muscles, however, appear to have a higher purine-content than those of other mammals studied [Truszkowski, 1927, p. 1043].

As to inanition, the values found are constant for all stages of hunger, this applying to total and purine-nitrogen contents and to the nuclear-plasmic ratio. A general tendency towards increase in the latter in hunger appears, however, in liver tissue. Truszkowski obtained similar results for dogs. The marked lack of constancy in the results does not allow, however, of the drawing of any more definite conclusion (Table II).

Table II. *Rabbit liver.*

No.	% solid substance	% N live wt.	% N dry wt.	Purine-N mg. per 100 g. live wt.	Purine-N mg. per 100 g. dry wt.	Nuclear-plasmic ratio	Remarks
I	27.5	2.58	9.39	121.4	441	$49.3 \times 10^{-3}$	Fed animals
II	24.6	3.12	12.68	161.4	655	$54.5 \times 10^{-3}$	"
III	26.8	2.75	10.26	137.2	528	$52.5 \times 10^{-3}$	"
IV	29.1	3.38	11.68	153.8	524	$47.7 \times 10^{-3}$	"
Mean	27.0	2.96	10.96	143.45	531	$50.92 \times 10^{-3}$	
VI	29.1	3.64	12.5	177.5	610	$51.3 \times 10^{-3}$	Inanition
VII	29.9	3.33	11.13	109.7	370	$34.1 \times 10^{-3}$	"
VIII	33.0	2.96	8.96	178.0	538	$64.0 \times 10^{-3}$	"
IX	28.6	3.67	13.10	145.0	520	$41.03 \times 10^{-3}$	"
XII	23.0	3.21	13.36	168.8	734	$55.5 \times 10^{-3}$	"
XIII	26.0	3.46	13.32	169.0	650	$51.3 \times 10^{-3}$	"
XIV	25.55	3.67	14.37	208.4	810	$60.2 \times 10^{-3}$	"
XV	26.0	4.16	16.01	140.3	539	$34.86 \times 10^{-3}$	"
Mean	22.64	3.51	12.70	162.1	586	$48.42 \times 10^{-3}$	

Previous extraction with water did not, with the exception of determinations 1 and 8, where the difference observed may be due to some experimental error, to any great extent affect the results obtained for the nuclear-plasmic ratio of unextracted muscle. The purine-nitrogen content whether of the muscles of fed or of starved animals was uniformly about half that found before extraction, whilst the total nitrogen content fell in all cases by about 15 %. The nuclear-plasmic ratio, although now expressed by a different figure (Table III), exhibited the same constancy with respect to different conditions of nutrition as did that of unextracted muscle.

Table III. *Extracted rabbit skeletal muscle.*

No.	% solid substance	% N live wt.	% N dry wt.	Purine-N mg. per 100 g. live wt.	Purine-N mg. per 100 g. dry wt.	Nuclear-plasmic ratio	Remarks
I ♀	25.0	3.212	12.84	70.40?	281?	$22.41 \times 10^{-3}$ ?	Fed animals
IV ♂	25.3	3.204	12.66	39.07	154	$12.03 \times 10^{-3}$	"
V	25.8	3.286	12.74	43.78	171	$13.68 \times 10^{-3}$	"
Mean	25.4	3.234	12.75	41.42	162	$12.85 \times 10^{-3}$	
VIII	23.75	3.17	13.40	74.19?	312?	$23.97 \times 10^{-3}$ ?	Inanition
IX	25.0	3.38	13.50	46.47	185	$14.37 \times 10^{-3}$	"
XI	27.8	3.86	12.08	39.28	141	$11.8 \times 10^{-3}$	"
XV	26.3	3.34	12.7	47.22	180	$14.3 \times 10^{-3}$	"
Mean	25.71	3.31	12.92	43.32	169	$13.29 \times 10^{-3}$	

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Similar results were obtained for the skeletal muscle of dogs, some of which had been fed for 3 months exclusively on oatmeal, whilst others were fed upon meat for 2 months. The same uniformity in the nuclear-plasmic ratio was found for yearling trout, starved for 6 months, and starved for 1 month, after which they were fed with liver for 5 months (cf. Table IV).

Table IV.

No.	% solid substance	% N live wt.	% N after extraction	After extraction				Nuclear-plasmic ratio after extraction	Remarks
				Purine-N mg. per 100 g. live wt.	Purine-N mg. per 100 g. dry wt.	Purine-N mg. per 100 g. live wt.	Purine-N mg. per 100 g. dry wt.		
1 ♂	—	3.50	3.0	79.13	—	30.61	—	23.12	Skeletal muscle of dog fed on oatmeal
2 ♂	26.2	3.71	3.10	79.37	303	32.32	123	21.80	Skeletal muscle of dog fed on meat
3	17.0	2.61	2.09	88.5	520	52.55	309	35.08	Whole trout, six months' inanition
4	20.17	2.92	2.20	88.7	440	50.35	250	31.3	Whole trout, one month's inanition, five months' restitution

## DISCUSSION.

The above results on the whole confirm those of Truszkowski [1927], who found that the nuclear-plasmic ratio of mammalian skeletal muscle remains constant throughout starvation. This would militate against the protein reserve theory on the assumption that the purine-nitrogen content of the cells remains unchanged. Whilst this might be conclusive evidence for many tissues, in which all or the great majority of purines present are of nuclear origin, it can in no case apply to muscular tissue, in which, as will be shown in a subsequent paper, the quantity of purines not belonging to the nucleus is exceptionally high, amounting to 50 % or more of the total purine content. Should, however, the total purine content remain constant during hunger, the interpretation of the constancy of the nuclear-plasmic ratio as evidence against the existence of a protein reserve would be justifiable. Since muscles, after extraction of all non-protein nitrogen derivatives and extramuscular purines, exhibit the same general constancy of nuclear-plasmic ratio as without extraction, it would seem that the above conclusions as to the absence of reserve protein in these tissues remain valid.

The parallelism existing between changes in total purine-nitrogen content and in nuclein-phosphorus content has been frequently observed. Thus Burian and Schur [1897] observed that the nuclein-phosphorus content of puppies increased 3.5 times during a period of growth over which the purine-nitrogen content increased 2.5–3 times. Masing's [1911] figures for the fall in nuclein-phosphorus content of rabbit foetuses with age correspond to those found by Schaeffer and Le Breton for purine-nitrogen, and Javillier's [1923] researches upon the nuclein-phosphorus content of mice during lactation are similarly in agreement with the results of these authors.

This parallelism is apparently due to the content of extranuclear purine substances rising together with the growth of tissues, and remaining constant after growth has ceased. These extranuclear purines cannot therefore be considered as being entirely independent of other cell constituents.

This constancy of extranuclear purine-nitrogen content is an extremely favourable factor, inasmuch as it enables one to make use of changes in the ratio  $N_p/(N_T - N_p)$  for the study of reserve protein. In those cases where considerable increase in this ratio is found, amounting in the case of trout to 40 % [Dmochowski, 1928], it is extremely improbable that the presence of extranuclear purine substances could in any way affect the conclusions drawn from this increase. Where, however, as for amphibia and mammalian muscle, this ratio remains constant or falls slightly, further examination of the extranuclear purine content would be necessary before conclusions as to the absence of reserve protein could be accepted without reserve.

#### SUMMARY.

1. The chemical nuclear-plasmic ratio of liver and skeletal muscles of rabbits, taken at various stages of total inanition up to the final stage, marked by the pre-mortal rise in nitrogen excretion, has been determined.

2. The purine-nitrogen content of rabbit skeletal muscle has an average value of 104.6 mg. per 100 g., varying from 85 to 119 mg.; this value is higher than that of other mammals studied.

3. The mean value of the nuclear-plasmic ratio is  $28.55 \times 10^{-3}$ , and this value remains constant throughout the duration of inanition. The same applies to the purine-nitrogen content.

4. The purine-nitrogen content of rabbit liver exhibits even more considerable individual variations than that of muscular tissue; it varies from 149.5 mg. per 100 g. in fed animals to 162.1 mg. in inanition. The mean nuclear-plasmic ratio is respectively 50.9 and  $48.4 \times 10^{-3}$  before and after starvation.

5. Over 50 % of the purines of muscle tissue are extracted by hot water, and are not therefore contained in the nucleus.

6. Parallel results were obtained on muscle from which extranuclear purine substances had been removed by extraction with hot water; in this case, too, the value found for the nuclear-plasmic ratio remains constant.

7. The agreement between change of nuclein-phosphorus content and purine-nitrogen content observed by a number of authors is probably due to the relatively constant content of extranuclear purine substances.

I would, in conclusion, desire to express my sincere gratitude to Prof. S. J. Przylecki for his never-failing advice and assistance.

REFERENCES.

- Burian and Schur (1897). *Z. physiol. Chem.* **23**, 55.  
 Caspari and Stilling (1925). Oppenheimer, *Handb. der Biochem.* **8**, 660.  
 Deuel, Sandiford, Sandiford and Boothby (1928). *J. Biol. Chem.* **76**, 391.  
 Dmochowski (1928). *Biochem. J.* **22**, 1548.  
 Embden and Zimmerman (1927, 1). *Klin. Woch.* **6**, 628.  
 ——— (1927, 2). *Z. physiol. Chem.* **167**, 137.  
 Javillier (1923). *Bull. Soc. Chim. Biol.* **7**, 778.  
 Kikuchi (1923). *Biochem. J.* **2**, 409.  
 Masing (1911). *Z. physiol. Chem.* **75**, 135.  
 Schaeffer and Le Breton (1923). *Trav. Inst. Physiol. Strasbourg*, 45, 57, 92.  
 Schultz (1899). *Arch. ges. Physiol.* **76**, 379.  
 ——— (1901). *Z. Biol.* **41**, 368.  
 Serio (1923). *Biochem. Z.* **142**, 440.  
 Truszkowski (1926). *Biochem. J.* **20**, 437.  
 ——— (1927). *Biochem. J.* **21**, 1040, 1047.  
 Voit (1901). *Z. Biol.* **41**, 570.





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